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Our original hypothesis was that breast tumor progression is more realistically modeled by a defect in cell-cell adhesion that results from an alteration in any one or more of the steps (molecules) required for E-cadherin function rather than loss of E-cadherin alone. This hypothesis has been borne out by our work over the past five years. In particular our discovery that increased expression of the mesenchymal cadherin 11 is associated with invasive breast cancer represents a paradigm shift in this area. We are now testing the effects of small molecule cadherin-11 disruptors. The past five years has also seen the expansion of the field to encompass the intracellular signaling and oncogenic activities of the cadherin-associated molecule β-catenin. Our contributions in this area include the discovery of a key role for β-catenin in the regulation of the cell cycle and contact inhibition. A recurring theme in the area of cancer treatment is the relationship between environmental influences such as diet and the molecular pathways that cause cancer. We have published several papers in this area and have demonstrated a direct relationship between the retinoid alpha receptor and β-catenin itself. These results have major implications in the area of cancer prevention.

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S.W. Page 1/7/00

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TABLE OF CONTENTS:

Introduction

Defects in cell-cell adhesion are commonly associated with tumor progression. There is evidence that alterations in the expression of the calcium-dependent cell adhesion molecule E-cadherin occur in a subset of invasive breast cancers and breast cancer cell lines. However, many invasive breast cancers and metastases are E-cadherin positive. Preliminary results indicate that breast tumor progression may more often be accompanied by alterations in the expression and function of several cadherin-associated molecules that are essential for cadherin-mediated cell-cell adhesion. It is the aim of this proposal to test the hypothesis that, in addition to the occasional loss of E-cadherin expression, breast tumor progression is more realistically modeled by a defect in cell-cell adhesion that results from an alteration in any one or more of the steps (molecules) required for E-cadherin function. We will take two fundamental approaches. Firstly, we will use two methods for "non-specifically" assessing E-cadherin function and cell-cell adhesive strength in breast tumor samples and cell lines. Secondly, we will specifically investigate the molecular mechanisms that lead to defects in cell-cell adhesion by examining (and manipulating) the expression and phosphorylation state of several E-cadherin associated molecules in breast tumors and cell lines.

Body

Task 1. To test the hypothesis that cell-cell adhesive strength and E-cadherin triton solubility is correlated with functional E-cadherin-mediated cell-cell adhesion (7,8,10-12).

During the period of support grant we refined our biophysical methods for measuring cell-cell adhesion strength and confirmed that the presence of detergent-insoluble E-cadherin or β-catenin correlates with strong cell cell adhesion. We also showed that treatment of cells with retinoic acid increased adhesion strength via increased cadherin function. In other studies we used our laminar flow assays to demonstrate a role for selectins and vinculin in cancer cell adhesion strength. This work is published and is summarized below (7,8,10-12).

Defects in the expression or function of the calcium dependent cell-cell adhesion molecule E-cadherin are common in invasive, metastatic carcinomas. In the present study the response of aggregates of breast epithelial cells and breast and colon carcinoma cells to forces imposed by laminar flow in a parallel plate flow channel was examined. Although E-cadherin negative tumor cells formed cell aggregates in the presence of calcium, these were significantly more likely than E-cadherin positive cell aggregates to disaggregate in response to low shear forces, such as those found in a lymphatic vessel or venule (<3.5 dyn/cm^2). E-cadherin positive normal breast epithelial cells and E-cadherin positive breast tumor cell aggregates could not be disaggregated when exposed to shear forces in excess of those found in arteries (> 100 dyn/cm^2). E-cadherin negative cancer cells, which had been transfected with E-cadherin exhibited large increases in adhesion strength only if the expressed protein was appropriately linked to the cytoskeleton. These results show that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. Since a primary route of dissemination of many carcinoma cells is to the local lymph nodes these results point to a novel mechanism whereby defects in cell-cell adhesion could lead to carcinoma cell dissemination.

Task 2. To measure the expression and phosphorylation state of cadherin-associated proteins in breast tumors and cell lines

Several publications arose as a result of support from the DOD in the area of phosphorylation of the cadherin-asociated proteins catenins in breast cancer cells. These data are summarized in publications included in the appendices (4,5, 9 and 10). Perhaps the most important observation was our demonstration that serine phosphorylation of the cadherin-associated oncogene β-catenin regulates its ubiquitination and degradation in an APC-dependent manner.

Task 3. Statistical analyses (years 3-4). Results will be correlated with tumor stage, blood vessel count, lymph node status, the expression of prognostic markers and period of metastasis-free survival. We are still accumulating data for this task and hope to submit this work in the next few months. Preliminary examination of the data points strongly to a significant association of cadherin 11 expression with breast cancers with a poor prognosis.

Task 4. To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength: We examined the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength by directly examining the effects of kinase inhibitors and plakoblobin transfection on cell-cell adhesion strength using biophysical methods.

Results showed that in two invasive cell lines β-catenin is constitutively heavily tyrosine phosphorylated. In this specific aim we tested the hypothesis that this hyperphosphorylation is the cause of the failure of transfected E-cadherin to alter the phenotype of the cells. We have found that only one of the cell lines (BT549) responds to tyrosine kinase inhibitors by alterations in E-cadherin-mediated adhesion. We have used several classes of kinase inhibitors the most effective being herbimycin A. The second cell line (HS578T) does not respond to any of the tyrosine kinase inhibitors even at very high doses. Unlike BT549 cells, which express low but detectable levels of the cadherin-associated molecule plakoglobin, HS578T cells do not express plakoglobin. We found that expression of plakoglobin in HS578T cells did not make the cells more responsive to E-cadherin transfection. We subsequently found that HS578T cells express N-cadherin and cadherin-11 and predict that it is the presence of these mesenchymal cadherins rather than low plakoglobin expression that is important in their invasive phenotype.

Approved Changes to the SOW:

1) Investigations of the mechanism whereby retinoic acid increases cell-cell adhesion strength and regulates β-catenin signaling (2, 3, 10).

In this study we showed that a breast cancer cell line (SKBR3) which expresses no E-cadherin very low levels of β-catenin protein and exhibits a poorly adhesive phenotype in Matrigel, responds to retinoic acid (RA) by a marked increase in epithelial differentiation. Specifically, treatment of cells with all trans RA, 9-cis RA or a RA receptor α-specific ligand resulted in a large increase in cell-cell adhesive strength and stimulated the formation of fused cell aggregates in Matrigel. A retinoid X receptor-specific ligand was ineffective. Exposure of cells to RA for

as little as 4 h was sufficient to maintain the adhesive phenotype for at least 4 days. The effects of RA required protein and RNA synthesis but were not mediated by factors secreted by stimulated cells, nor by direct cell contact and did not require serum. These RA-induced morphological effects were completely reversed by growing cells in 50 μM Ca^{++} suggesting a mechanism involving a RA-induced increase in Ca^{++}-dependent adhesion. Consistent with this, β-catenin protein levels were markedly elevated in the RA-treated cells and β-catenin became localized to a Triton-insoluble pool at regions of cell-cell contact. No change could be detected in β-catenin steady state mRNA levels but RA did increase β-catenin protein stability. Treatment of cells with low calcium medium did not prevent the RA-induced increase in total β-catenin protein but did prevent its movement to a Triton-insoluble pool at the cell membrane. Among several kinase inhibitors, only the broad-spectrum kinase inhibitor staurosporine and the protein kinase C inhibitor bisindoylmaleimide reversed the morphological changes induced by RA. Like treatment with low calcium medium, these inhibitors did not prevent the RA-induced increase in total β-catenin protein levels but completely prevented the movement of β-catenin to the cell membrane. These results, point to a role for β-catenin and serine kinase activity in mediating the action of RA in epithelial differentiation.

Other results show that in addition to its epithelial-differentiation properties, retinoic acid can inhibit the signaling activity of cytoplasmic β-catenin/LEF (3). Preliminary results indicate that the effects of retinoic acid are mediated directly at the level of β-catenin/LEF transactivation. This is very significant because it might point to a new and perhaps general mechanism whereby retinoids affect differentiation and proliferation.

2) Investigations of the mesenchymal cadherins, cadherin 11 and N-cadherin (6). In this work we showed that expression of the mesenchymal cadherins, cadherin 11 and N-cadherin is restricted to invasive breast cancer cells. The implications of this work are significant. Since cadherin-11 is not expressed on normal epithelial cells or on non-invasive tumor cells it could provide a target for therapies directed at interfering with its function.

3) Role of β-catenin in proliferation and contact inhibition (5): Our data show directly for the first time that β-catenin itself has transforming properties. In keeping with a role for cell adhesion in this process we found that β-catenin influenced the process of contact inhibition rather than growth per se as well as allowing cells to grow in soft agar. β-catenin also inhibited cells from undergoing suspension-induced apoptosis (anoikis) and made cells resistance to radiation-induced growth arrest. This work is significant because it demonstrates a molecular basis for the transforming effects of wnt and APC mutation in breast cancer and provides a mechanism for contact inhibition.

Key Research Accomplishments

In this final report I will summarize the results of the work supported by the grant. Detailed reports can be found in the previous annual reports and above. 10 papers have been published and several more are submitted or in preparation. These are listed under "Reportable Outcomes".

1. Biophysical measurements of adhesion demonstrate a role for changes in the strength of cell-cell adhesion in regulating breast cancer metastasis.
2. Serine phosphorylation of the cadherin-associated oncogene β-catenin regulates its ubiquitination and degradation in an APC-dependent manner.
3. β-catenin is a key element in the processes of contact inhibition and cell cycle regulation.
4. Interactions between the chemopreventive agent vitamin A and β-catenin occur and may have major clinical significance.
5. Cadherin-11 is a marker for invasive breast cancer
6. A second APC gene, APC2 has been identified and is deleted in a significant number of primary breast cancers.

Reportable Outcomes

Manuscripts, abstracts, presentations

1.Jarrett, C., Young, P., Haddad, B., King, C.R. and Byers, S. Chromosomal fine mapping, localization and regulation of human APC2. Submitted

2. Pishvaian, M., Easwaran, V, Brown, P. and Byers, S. The role of cadherin, β-catenin and AP-1 in retinoid regulated breast cancer cell differentiation and proliferation. Submitted

3. Easwaran,V., Pishvaian, M., Salimuddin and Byers, S. (1999) Cross-regulation of β-catenin TCF/LEF and retinoid signaling pathways. Current Biology 9: 1415-1418

4. Easwaran,V. Song, V., Polakis, P. and Byers, S. (1999) The ubiquitin-proteosome pathway and serine kinase activity regulate APC modulation of β-catenin/LEF signaling. J. Biol Chem 274: 16641-16645.

5. Orford, K., Orford, C. and Byers, S. (1999) β-catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell cycle arrest. J.Cell.Biol. 146: 1-14

6. Pishvaian, M., Feltes, C., Thompson, P. and Byers, S. (1999) Expression of the mesenchymal cell-adhesion molecule cadherin 11 associated with invasive breast cancer. Cancer Research, 59; 947-952

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Keith Orford MD.PhD 2001- DOD fellow, supplies provided by 5012
Mike Pishvaian MD.PhD 2001- DOD fellow, supplies provided by 5012
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Carolyn Feltes MD.PhD 2002- DOD fellow, supplies provided by 5012
Christy Jarrett PhD 2001- DOD fellow, supplies provided by 5012

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Pending
Mesenchymal cadherins and breast cancer. DOD. PI Stephen Byers (25% effort). FYDC $112,701
β-catenin and cancer. NIH. PI Stephen Byers (25% effort).
Genetics and cell biology of APC2. NIH. PI Stephen Byers (25% effort).
Cross regulation of β-catenin and retinoid signaling. NIH. PI Stephen Byers (25% effort).

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Stephen Byers
Aydin Tozeren
Becky Hoxter
Keith Orford (until awarded fellowship)
Vijay Easwaran

Conclusions

Several major conclusions can be drawn from the work supported by DAMD17-1-95-5012.

1. Our original hypothesis was that breast tumor progression is more realistically modeled by a defect in cell-cell adhesion that results from an alteration in any one or more of the steps (molecules) required for E-cadherin function rather than loss of E-cadherin alone. This hypothesis has been borne out by our work and that of others over the past five years. In particular our discovery that increased expression of the mesenchymal cadherin 11 is associated with invasive breast cancer represents a paradigm shift in this area. In collaboration with industry we are now testing the effects of small molecule cadherin-11 disruptors.

2. The past five years has also seen the expansion of the field to encompass the intracellular signaling and oncogenic activities of the cadherin-associated molecule β-catenin. Our contributions in this area include the discovery of a key role for β-catenin in the regulation of the cell cycle and contact inhibition.
3. A recurring theme in the area of cancer treatment is the relationship between environmental influences such as diet and the molecular pathways that cause cancer. We have published or submitted several papers in this area and have recently demonstrated a direct relationship between the retinoic acid alpha receptor, and β-catenin itself. These results have major implications in the area of cancer prevention.

Appendices (manuscripts and papers not previously submitted)

Jarrett, C., Young, P., Haddad, B., King, C.R. and Byers, S. Chromosomal fine mapping, localization and regulation of human APC2. Submitted

Pishvaian, M., Easwaran, V, Brown, P. and Byers, S. The role of cadherin, β-catenin and AP-1 in retinoid regulated breast cancer cell differentiation and proliferation. Submitted

Easwaran,V., Pishvaian, M., Salimuddin and Byers, S. (1999) Cross-regulation of β-catenin TCF/LEF and retinoid signaling pathways. Current Biology 9: 1415-1418

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Chromosomal fine mapping, localization, and regulation of human APC2

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ABSTRACT

A second adenomatous polyposis coli (APC)-like gene, APC2 was recently described and localized to chromosome 19. We have now fine mapped APC2 to a small region of chromosome 19p13.3 containing markers D19S883 and WI-19632, a region commonly lost in a variety of cancers. APC2 is expressed in many different tissues and cell lines including brain, breast, colon, and ovary. Endogenous APC2 is diffusely distributed in the cytoplasm and co-localizes with both the Golgi apparatus and actin filaments. Unlike APC, APC2 and β-catenin remained associated with actin filaments following treatment with the actin-disrupting agent, cytochalasin D. In addition, APC2 co-localizes with β-catenin and actin filaments at the membrane of SKBR3 cells upon retinoic acid treatment. Like APC, APC2 has the ability to down-regulate β-catenin signaling and is sensitive to the PKC inhibitor bisindoylmaleimide. APC2 is more sensitive than APC to inhibition of GSK3 with LiCl and, unlike APC, can inhibit the signaling activity of a S37A mutant form of β-catenin. These results suggest that APC2 is involved in actin associated events and could influence cell motility through interaction with actin filaments as well as functioning independently or in cooperation with APC to down-regulate β-catenin signaling.

INTRODUCTION

The APC tumor suppressor gene, located on chromosome 5q21, is associated with colon cancer. Possible functions include the regulation of β-catenin protein degradation and signaling and microtubule mediated cell migration (27;30;33). β-catenin binds to the Tcf/LEF transcription factor complex and regulates the transcription of c-myc and cyclin D1, thus indicating that this pathway may be involved in cell cycle regulation (17;22;32;37;43). Truncating mutations in APC or mutations in certain N-terminal serine residues of β-catenin, result in increased β-catenin levels and increased transcriptional activation (26;27;31;33).

APC is a large protein, approximately 320 kD, containing many different domains including an N-terminal dimerization domain, a conserved domain of unknown function, armadillo repeats, β-catenin binding and regulation domains, axin binding domains, a microtubule binding domain, and a human discs large (HDLG) binding domain (33). A search for APC-like genes in the HGS/TIGR (Human Genome Sciences, Inc., and The Institute for Genomic Research) proprietary database of human expressed sequence tags (ESTs) resulted in the identification of an N-terminal sequence with significant homology to the human, mouse, frog, worm, and fly APC genes. During the course of our work, two studies published the full-length sequence of this gene as APCL and APC2 (29;45). Like APC, APCL/APC2 interacts with β-catenin and can decrease β-catenin levels and signaling activity in SW480 colon cancer cells (29). A second APC-like gene has also been identified in Drosophila (12;24;51). We now show that this gene, which we will call APC2, is located close to markers D19S883 and WI-19632 on a region of chromosome 19p13.3 commonly lost in a variety of cancers (3;7;23). APC2 is expressed in many different tissues and cell lines including brain, breast, colon, and

ovary. Importantly, APC2 is expressed in many of the same tissues and cell lines as APC, indicating a non-redundant function. Antibodies against the N-terminal of human APC2 detect endogenous APC2 associated with the Golgi apparatus and actin filaments, particularly those filaments present at the leading edge of the cell and at cell-cell contact sites. Like APC, APC2 has the ability to inhibit β-catenin signaling and is sensitive to the PKC inhibitor bisindoylmaleimide. APC2 is more sensitive than APC to inhibition of GSK3 with LiCl and, unlike APC, can inhibit the signaling activity of a S37A mutant form of β-catenin.

MATERIALS AND METHODS

Identification and DNA sequencing of APC2: A homology search was performed using the human APC sequence against the HGS/TIGR (The Institute for Genomic Research and Human Genome Sciences, Rockville, MD) proprietary database of human expressed sequence tags (ESTs) (1;2). In 1997, an APC-like EST was identified from a human infant brain cDNA library, and the corresponding cDNA clone was recovered. DNA sequencing was carried out on both strands of the cDNA clone by using an automated ABI 373 DNA Analysis System (Applied Biosystems). RNA was isolated from SKBR3 cells by the RNazol method (Tel-Test, Inc.). The RNA served as template in RT-PCR reactions using sequence-specific primers and the Expand RT-PCR System (Boehringer-Mannheim), according to manufacturer's protocol. The amplified product was subcloned using a TA Cloning kit (Invitrogen). TA cloned RT-PCR products were sequenced as described above.

FISH Analysis and Fine Mapping: A 1kb cDNA fragment from the N-terminal region of APC2 corresponding to the recombinant protein used to make antibodies was used to screen a P-1 derived artificial chromosome (PAC) library (Human Genome FISH Mapping Resource Centre at the Ontario Cancer Institute). Four genomic PAC cloness were identified: 1K8, 17J21, 22K8, and 26K20. Fluorescence in situ hybridization (FISH) to normal human lymphocyte chromosomes was used to map the genomic PAC clones to chromosome 19p13.3. Fine mapping was performed using radiation hybrid screening by PCR (Research Genetics, Inc.). Primer sequences (5′-GCTGCAGGAGCTGAAGATG; 5′-GTGGCTGGAGTTGTCCCTTA) were designed to yield a 120 bp product spanning the first exon/intron junction.

Antibody Development: A recombinant GST-fusion protein to the N-terminal region of APC2 (aa 1- 249) was produced in *E. coli* using the pGEX-4T-2 vector, isolated, and released by protease cleavage (Pharmacia Biotech). This protein was used to inoculate both rabbit and chicken (Rockland Inc., Gilbertsville, PA). Both the rabbit serum and IgY collected from the chicken eggs was affinity purified on an antigen coupled CnBr column (XMMR website at http://vize222.zo.utexas.edu/Marker_pages/methods_pages/affinity_col.html).

Northern Analysis: Human multiple tissue and human cancer cell line poly(A)+ RNA blots were obtained from Clontech and processed according to the supplied manufacturer's protocol using a probe to the N-terminal region of APC2.

RT-PCR: RNA was isolated by the RNAzol method (Tel-Test, Inc.). RT-PCR was performed using the Perkin-Elmer Gene Amp RNA PCR Core Kit. Primers to the N-terminal region (5′-AGGAGCTAAGGGACAACTCCA; 5′-TCCAGCAGCTCCTTGTCAAT) were designed to yield a 600 bp fragment. These primers were shown to be specific to APC2 by sequencing of the product by the above method as well as using wt-APC as a negative control.

Western Blot: Cells were grown to confluence in 150 mm dishes, washed twice with phosphate buffered saline (PBS) and lysed for 10 minutes on ice in 1% HEPES lysis buffer containing 1% Triton-X and protease inhibitors (1 mM sodium vanadate, 50 mM sodium fluoride, and Boehringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet). Lysates were centrifuged at 14,000 rpm at 4°C. Protein content was determined by the BCA protein assay (Pierce). Cytoplasmic and detergent soluble and insoluble fractions were made as

described previously (31). Western blotting was performed as previously described using either rabbit or chicken APC2 antibody at 1 μg/ml, APC Ab-1 (Oncogene) at 1 μg/ml, or β-catenin (Transduction Laboratories) at 1:1000 (40). The blots were developed using chemiluminescent detection (Pierce). Specificity of the antibodies was determined by incubating recombinant APC2 antigen (10 μg/ml) with the antibody for 1 hour at room temperature before incubating the blot.

Immunocytochemistry: SKBR3, A549, MDA-MB-157, SW480, and MDCK cells were plated on 18 mm coverslips in 12 well plates at approximately 100,000 cells/well. In some experiments, SKBR3 cells were treated with 10^{-6} M retinoic acid (RA) for 24 hours. In other experiments, cells were treated with 2 μM cytochalasin D (Sigma) in media for 2 hours at 37°C. Antibody blocking with the immunogen was performed as described above. Both treated and untreated cells were fixed with 2% paraformaldehyde and permeablized with 0.2% Triton. Purified chicken APC2 antibody was used at a concentration of 1 μg/ml and secondary antibody conjugated with fluorescein (Pierce) was used at 1:100 while secondary antibody conjugated with Texas Red (Rockland) was used at 1:150. Other primary antibodies and reagents were used at the following concentrations: normal IgY (Rockland) at 1 μg/ml, monoclonal β-catenin antibody (Transduction Laboratory) at 1:100 overnight at 4°C, polyclonal anti-APC (kindly provided by P. Polakis (27)) at 1:100 overnight at 4°C, monoclonal anti-tubulin (Sigma) at 1:2000, phalloidin (Molecular Probes, Inc) at 1:200 for 15 minutes, anti-PKCμ (Transduction Lab.) at 1:200, monoclonal anti-EEA1 (Transduction) at 1:1000, and LysoTracker Red (Molecular Probes, Inc.) at 0.1 μM in media for 3 hour incubation at 37°C (cells were not permeabilized when using LysoTracker Red). All primary antibodies were incubated for 1 hour

and all secondary antibodies were used at a 1:100 dilution for 1 hour at room temperature unless otherwise noted above.

LEF Reporter Assay: SW480 cells were plated at ~100,000 cells/well in a 12 well plate. After 24 hours, the cells were transfected using the Lipofectamine Plus (GibcoBRL) method with either a control PCDNA3-cat expression vector, APC2 full length cDNA (kindly provided by Y. Nakamura), or wild-type APC (0.1 μg each) along with renilla (2 ng) and TopFlash (0.1 μg) (44). Wild-type and S37A β-catenin (0.1 μg) were transfected with 0.3 μg of APC or APC2. All transfections were done in triplicate and repeated at least three times with the LEF reporter activity measured in lumens after 48 hours using the luciferase assay (Promega). Cells were treated as indicated 12-16 hours after transfection and collected 36 hours later, with the exception of LiCl which was added with fresh media 3 hours after transfection and collected 48 hours later. A dose response was also performed with each treatment and the optimal doses chosen for these experiments.

RESULTS

Identification of a Novel APC-Like Gene. We isolated a 1364 bp sequence from a human infant brain cDNA library by screening of the HGS/TIGR proprietary EST database. This partial sequence was 49% identical to human APC and contained an N-terminal dimerization domain. During the course of this work, a similar sequence was submitted to the public EST database. Shortly thereafter the full-length cDNA sequence was published as both APCL and APC2 (45;51). Figure 1A illustrates the domain structure of all known members of the APC family. A number of conserved domains are variably present in APC family members. The best studied of these, human APC has the following domains (33): 1. an N-terminal domain that can mediate dimer formation between two APC monomers (19;42), 2. a conserved domain of unknown function, 3. seven armadillo repeats, which in other proteins are thought to mediate protein-protein interactions, 4. three 15 amino acid repeats, which can bind β-catenin constitutively, 5. seven 20 amino acid repeats, which can bind and target β-catenin for degradation: 6. three SAMP repeats, which can interact with axin (6), 7. a basic domain with microtubule binding properties: 8. a discs large binding site at the extreme C-terminal. Mouse and xenopus APCs have very similar structures (not shown). None of the other APC genes has a disks large binding site. Overall hAPC2 is 35% identical to human APC and the protein product predicted to be slightly smaller than APC (245 kD vs 310 kD). However, the N-terminal region has much greater homology to APC than the C-terminal. The N-terminal dimerization domain has 68% identity to APC, the conserved domain 45%, the armadillo repeat region 76%, and the β-catenin binding region greater than 50% (Figure 1). The C-terminal region of APC is only 30% conserved in APC2. APC2 lacks the three 15-amino acid constitutive β-catenin binding repeats and contains only five of the seven 20 amino acid repeats. The three axin binding SAMP

repeats in APC are poorly conserved in hAPC2. However, the SAMP repeats in mAPC2 can bind axin/conductin (45).

APC2 is more closely related to human APC than are Drosophila APC (dAPC) and the C. elegans APC related gene (ARG). dAPC does not contain an N-terminal dimerization domain or microtubule binding domain, but does contain the conserved domain, the armadillo repeats, one 15-amino acid repeat, the 20-amino acid repeats, and the basic region (16). ARG, however, only contains the armadillo repeats and two regions similar to the APC SAMP repeats (34). A second drosophila APC gene, APC2/E-APC (19% identical to hAPC2), is more similar to the dAPC gene (26% identical) in structure except that it is much smaller and is missing three of the 20 amino acid repeats as well as the basic domain (12;51;52). In addition, Drosophila APC2/E-APC differs significantly from human and mouse APC2 in that it is missing the dimerization domain and has retained two of the 15 amino acid constitutive binding β-catenin repeats.

Chromosomal Localization and Fine Mapping. Using a 1 kb sequence to the N-terminal region, four PAC clones (1K8, 17J21, 22K8, and 26K20) were isolated for APC2. FISH analysis using the four clones localized APC2 to chromosome 19p13.3, which confirms the previously published chromosomal assignment (7,8). 19p13.3 is ~20 mb in size. The genomic sequence of APC2 is ~ 40 kb and the coding sequence 7 kb. APC2 was then fine mapped by radiation hybrid mapping to the 800 kb region containing markers D19S883 and WI-19632 using primers designed to span the first exon/intron junction (Figure 1B). This particular region of 19p13.3 exhibits significant loss of heterozygosity (LOH) in many different cancers and is near the Peutz-Jeghers syndrome (PJS) associated gene, LKB1/STK11. PJS is characterized by intestinal

hamartomas and increased risk of gastrointestinal, ovarian, pancreatic, and breast cancers (25). Even though there is significant LOH in this region, there are few mutations in the LKB1 gene in sporadic breast and colorectal cancers and adenoma malignum of PJS patients (3;7). In addition, although 50% of ovarian cancers contain on LOH on 19p13.3, LKB1 is not mutated indicating that another gene of significance in the development of cancer exists in this region (46). Marker D19S216, which is 9.5 fcM distal to marker D19S883, but not LKB1 itself, exhibits 100% LOH in sporadic adenoma malignum of the uterine cervix (23). Therefore, APC2 could be a tumor suppressor gene affected by LOH of chromosome 19p13.3.

APC2 Expression. APC2 expression was determined by RT-PCR and Northern analysis of both cell lines and tissue. APC2 was expressed in a variety of cells and tissues, including breast, colon, brain, and ovary, at both the RNA and protein level (Table I). APC2 expression, like APC, is greatest in the brain; however, there are differing levels in different brain regions with very little expression in the cerebellum and cerebral cortex (Figure 2A). Lymphoid tissues and lymphoma cell lines had no detectable APC2 at the mRNA or protein level with the exception of K-562 leukemia cells, which express low levels of APC2.

Both rabbit and chicken hAPC2 antibodies were affinity purified on an antigen coupled CNBr column. Western blot analysis determined that both antibodies were specific to hAPC2 with no cross-reactivity to APC (Figure 2B). This was confirmed using the SW480 colon cancer cell line that contains a C-terminal truncated form of APC. Neither rabbit nor chicken hAPC2 antibodies detect this truncated APC protein even though it contains the conserved N-terminal. The largest form of APC2 is slightly smaller than APC in the HBL-100 and MDA-MB-468

breast cancer cell lines and corresponds to the predicted 245 kD molecular weight (Figure 2B). To further determine specificity, we blocked the antibody with recombinant antigen before western blot analysis and found that all bands are specific to APC2 (not shown). Western blot analysis showed that APC2 is expressed in many cells lines including SKBR3, SW480, MDCK, MDA-MB-157 and 436 (Figure 2C). A characteristic pattern of immunoreactive species was observed. Three major bands larger than 200 kD and several smaller molecular weight species of~121, 81, and 51 kD (not shown on this blot) were present consistently. Most cell lines express the three >200 kD species with varying levels of the smaller species (compare 121 kD band of SKBR3 with MDA-MB-436 and SW480). Other cell lines, for example MDA-MB-157, have significantly less of all bands except for two >200 kD species. The presence of multiple bands by western blotting with APC2 antibodies is similar to that observed with APC antibodies (36). In the case of APC, these bands most likely represent degradation products or some of the 16 known splice variants.

Sub-cellular Localization of APC2. To investigate the localization of APC2 in the cell, we performed immunocytochemistry on several cell lines including SKBR3, MDCK, SW480, MDA-MB-157, and A549 lung carcinoma cells. MDCK cells are derived from the kidney of a normal canine and had been used in previous studies of APC (30). SKBR3 and MDA-MB-157 cells are breast cancer cell lines. Although both rabbit and chicken antibodies exhibited a similar staining pattern by immunocytochemistry, the chicken antibody was exceptional and was used for these studies. Preimmune chicken IgY and antigen blocked antibody, as well as IgY prior to antigen affinity purification, were completely negative (Figure 3 A and B). Specific APC2 staining was similar in all cell lines and was observed diffusely in the cytoplasm as well as being

associated with tubular structures adjacent to the nucleus that resembled the Golgi apparatus (Figure 3). Staining was also concentrated along filamentous structures and in what appeared to be lamellipodial membranes.

To confirm the localization of APC2 to the Golgi apparatus, cells were double-stained with anti-APC2 and PKCμ, a kinase known to associate with the Golgi (18). Co-localization of APC2 and PKCμ was observed for much of the Golgi stack indicating that APC2 is associated with certain regions of the Golgi where it co-localizes with PKCμ (Figure 4 A1-3). APC2 staining is also associated with small vesicles/particles; however, staining with LysoTracker Red and the early endomsomal marker antibody, EEA1, eliminated lysosomes and endosomes respectively (results not shown).

APC2 Association with Actin Filaments. A relationship between APC2 and actin filaments was observed in cells stained with phalloidin. (Figure 4 B1-3). However, not all actin filaments stained and APC2 appeared to be concentrated near actin-associated membrane ruffles and lamellipodia as well as cell-cell contact sites (also see Figure 3). This staining pattern was similar for all but one of cell types tested. MDA-MB-157 cells had more pronounced actin-associated APC2 staining throughout the cell and less at cell-cell contact sites. Treatment with cytochalasin D, an actin disrupting agent, causes actin filaments to retract into clumps or balls mostly at the cell periphery but also throughout the cell. Following treatment with cytochalasin D, APC2 remained associated with the actin filaments in A549 cells (Figure 4 C1-3) and in MDA-MB-157 cells (Figure 4 D1-3).

Endogenous APC is localized at the tips of microtubules in MDCK cells and is not associated with actin filaments (30). However, overexpression of APC results in the decoration of microtubules throughout the cell (28;38). Consistent with this, cytochalasin D treatment did not affect APC staining but disruption of microtubules with nocodazole did (30). We confirmed that APC distrubution was unaffected by cytochalasin D treatment (not shown). Similarly, APC2 did not co-localize with microtubules in cells double-stained for tubulin and APC2 (results not shown). In addition, APC and APC2 were not co-localized in the cell. Even though APC and APC2 can be found concentrated at the cell membrane and often in the same general area, co-localization is not detected upon double-staining (results not shown). However, it should be noted that both APC2 and APC are present in the cytoplasm.

APC2 Association with and Regulation of β-catenin. One function of APC is the regulation of β-catenin function and/or turnover. However endogenous wild-type β-catenin and APC do not co-localize or do so only transiently (30). If an N-terminal truncated stable form of β-catenin is expressed in MDCK cells, then β-catenin can be found co-localized with APC (5). We next wanted to investigate if β-catenin and APC2 co-localized in cells. Like APC2, some β-catenin staining is associated with actin filaments (Figure 4 E1-3). Upon cytochalasin D treatment, β-catenin staining is disrupted and remains associated with actin filaments in a pattern similar to that observed for APC2 (Figure 4 F1-3). To address if APC2, which also has β-catenin binding and regulation domains, was localized to β-catenin-associated structures we treated SKBR3 cells with 10^{-6} M RA for 24 hours. We showed previously that SKBR3 cells have very low levels of β-catenin protein (39). However, after treatment with RA β-catenin levels increase markedly and it becomes localized to the membrane and to cell-cell contact sites (see Figure 5 C2 and D2;

(8)). After treatment of SKBR3 cells with RA, APC2 also localized to cell-cell contact sites and along the membrane in close association with β-catenin (compare Figure 5 A2 and B2). However, APC2 has more of a punctate staining pattern than β-catenin.

SKBR3 cells treated with RA change morphology and actin filaments become concentrated along the membrane and cell-cell contact sites (Figure 5 A1, B1, C1, and D1; (9)). APC2 co-localizes with actin filaments in both untreated and treated SKBR3 cells (Figure 5 A1-3 and B1-3). After RA-treatment APC2 is found concentrated at the membrane at the leading edge of the cell adjacent to but not precisely co-localized with actin filaments. At cell-cell contact sites APC2 and actin filaments are more precisely co-localized (Figure 5 B1-3). Untreated SKBR3 cells have very little, diffuse β-catenin staining, which is not associated with actin filaments (Figure 5 C1-3); however, after RA treatment regions of co-localization between actin and β-catenin can be found (Figure 5 D1-3). In untreated SKBR3 cells, only a few regions of co-localization can be found between APC2 and β-catenin as β-catenin levels are so low (see Figure 5 C2); however, after treatment APC2 and β-catenin association can clearly be seen at the membrane and especially at cell-cell contact sites (Figure 5 E1-3). APC2 also co-localized with β-catenin in untreated MDCK and A549 cells (results not shown). These cells, unlike SKBR3 cells, express β-catenin at the membrane in the absence of RA.

Cell fractionation shows that APC2 is primarily in the cytoplasmic fraction in both A549 and SKBR3 cells (Figure 6A and B). Based on our immunocytochemistry, we hypothesized that after RA treatment APC2 would move to the membrane fraction of SKBR3 cells. However, this is not the case (Figure 6B). To compare the amount of APC2 in the different fractions, equal cell

equivalents of each fraction instead of equal amounts of protein were loaded. APC2 remains concentrated in the cytoplasmic fraction whereas β-catenin clearly moves to the membrane fraction of RA treated SKBR3 cells. This result indicates that APC2 does not associate tightly with the membrane or β-catenin at the membrane. However, it is likely that our extraction conditions do not preserve a transient or low affinity interaction. That is, the change in localization of APC2 noted by immunocytochemistry might not be detectable after cell-fractionation. However, the RA treatment does alter the apparent molecular weight of the three high molecular weight species of APC2 in the detergent soluble fraction but not in the cytoplasmic fraction (Bands 1, 2, and 3). There is also significantly less of the splice variant/degradation products at ~51 K (pair of bands denoted by the arrowhead) in the RA treated cytoplasmic fraction compared to the untreated fraction.

Finally, we looked at the ability of APC2 to regulate β-catenin signaling. As shown previously, APC2 can inhibit β-catenin signaling in SW480 cells (Figure 7; (29;45)). Several studies have pointed to a role of PKC-like enzymes in the transmission of the wingless signal (10). We investigated the effects of Calphostin C, a diacylglycerol (DAG)-dependent protein kinase C (PKC) inhibitor, and bisindoylmaleimide (bis), which inhibits both DAG-dependent and independent PKC isoforms, on the ability of APC2 to inhibit β-catenin signaling. Calphostin C had little effect on APC2 inhibition of β-catenin signaling (Figure 7A). This is consistent with our earlier work in which we showed that this inhibitor did not increase cytoplasmic β-catenin. (31). In contrast, bis almost completely reversed the APC2 mediated inhibition of β-catenin signaling (Figure 7A). Bis also inhibits APC activity and increases cytoplasmic β-catenin

(11;31). These results point to a role for atypical PKC-activity in the regulation of both APC and APC2 function.

GSK3β forms a complex with axin, β-catenin, and APC, which can then regulate β-catenin turnover (20). Although the precise role of GSK3β is not clear, Li^+, which inhibits GSK3β activity, leads to the accumulation of β-catenin in the cytoplasm (31;41). We recently showed that LiCl does not significantly inhibit the ability of APC to down-regulate β-catenin signaling (11). In the present study we found APC2 to be somewhat more sensitive to LiCl than APC (Figure 7B). LiCl increases signaling 3 fold over APC2 alone compared to ~1.5 fold for APC. Another significant difference between APC and APC2 is the ability of APC2 to inhibit the signaling activity of a mutant S37A form of β-catenin that is resistant to inhibition by APC (Figure 7B; (11)).

DISCUSSION

The APC-like gene we isolated is identical to the recently published APCL and APC2 sequences (29;45). However, our data show that this gene is not brain specific, as Nakagawa et al. reported, but is found in many different tissues including breast, ovary, brain, and colon as shown by Western blotting, RT-PCR, and Northern analysis. However, like APC there is considerably more APC2 in the brain than most other tissues.

Relationship of hAPC2 to other APC family members

APC2 is more closely related to the human APC gene than the Drosophila APC gene or C. elegans ARG gene. Human APC2 does not contain the three 15 amino acid repeats thought to constitutively bind β-catenin, or the HDLG binding sites, and, therefore, should not be able to constitutively bind β-catenin or bind the discs large protein. APC2 does contain five 20 amino acid repeats that, in APC, are involved in the down-regulation of β-catenin protein and signaling activity and is able to inhibit β-catenin/LEF/TCF reporter activity (35). The axin binding SAMP repeats of APC are poorly conserved in hAPC2. Mouse APC2 SAMP repeats are more similar to those found in APC (notably the presence of M in the SAMP repeat) and can bind axin (45). hAPC2 contains AAVP and SALP instead of SAMP. Because a mutated SAMP (AALP) cannot bind conductin it is possible that these sites in hAPC2 may not bind axin (6). The differences between human and mouse APC2 genes in this region indicate either that they have a different function or that axin binding is not required for human APC2 to function. It is also possible that

other axin/conductin-like genes exist which exhibit different specificities for the various APC forms. A second drosophila APC gene does not contain the SAMP repeats but does contain two constitutive β-catenin binding sites. dAPC2 is similar in structure to dAPC except it is missing the C-terminal region. The relationship of hAPC2 to the drosophila APC2/E-APC is not clear. dAPC2/E-APC is not closely related to hAPC2 in terms of sequence similarity. Taken together with the existence of many, tissue specific splice variants of at least one APC gene, it is likely that the various members of the APC family have multiple tissue and context-dependent functions.

Significance of the chromosomal location of APC2

The chromosomal localization of APC2 to chromosome 19p13.3 is significant because this region is associated with Peutz-Jeghers Syndrome (PJS) and exhibits significant loss of heterozygosity (LOH) in several sporadic cancers. Patients with PJS are more susceptible to breast, testis, gastrointestinal, and ovarian cancers (25). Loss of 19p13.3 occurs in many sporadic cancers including those of the breast and is remarkably common in sporadic ovarian carcinomas (~50%) (46). Ovarian cancers are also characterized by a high rate (~16%) of stabilizing β-catenin mutations (48). However, mutations in the PJS gene, LKB1, are not present in most of these sporadic cancers suggesting the existence of other tumor suppressor loci in this region of chromosome 19 (7;46). Our fine-mapping analysis shows that APC2 is located in the region of markers D19S883 and WI-19632 between the LKB1 gene and the site of 100% LOH found in adenoma malignum of the uterine cervix (23). Therefore, like APC, APC2 could be a tumor suppressor gene important in several cancers.

Subcellular localization of hAPC2

hAPC2 is diffusely distributed in the cytoplasm, is localized to the Golgi apparatus, and is associated with actin filaments. In some instances, such as lamellapodia or membrane ruffles, APC2 exhibits a punctate staining at the ends of actin filaments. Unlike APC, APC2 remains associated with the disrupted actin filaments following treatment with cytochalasin D. β-catenin also remains associated with actin filaments following this treatment, indicating a close association with APC2. APC2 co-localizes with β-catenin and actin filaments at the plasma membrane, and in well differentiated cells such as MDCK and RA-treated SKBR3 cells, at cell-cell contact sites. Recent studies show that E-APC/dAPC2 co-localizes with actin caps during Drosophila development and negatively regulates wingless signaling in the epidermis (14;24;52). These data suggest that even though sequence similarity is low, hAPC2 and dAPC2/E-APC may be functional homologues and that both may be involved in actin-associated events such as motility as well as in β-catenin signaling. In contrast, endogenous APC localizes near the ends of microtubules in a punctate pattern but does not associate with actin (30). Upon overexpression, APC associates with microtubules throughout the cell (28;38). It has been suggested that APC might be involved in microtubule regulated membrane protrusion and cell migration as well as inhibition of β-catenin signaling (4). The present study demonstrates that APC2 and APC are present in the same cells indicating that they are not precise functional homologues and have non-redundant roles. Although APC2 and APC do not co-localize at the membrane or cytoskeletal structures they are both present in the cytoplasm. Preliminary results show that they can exist in the same complex in this environment (results not shown). Taken together these findings suggest an intriguing scenario in which cytoplasmic APC and APC2

regulate related microtubule and actin-based functions and β-catenin signaling either independently or in co-operation.

APC2 regulation of β-catenin signaling

Like APC, APC2 also can inhibit β-catenin signaling. However, this activity of APC and APC2 is regulated somewhat differently. Both proteins are equally susceptible to inhibition of atypical PKCs and both are equally resistant to inhibitors of DAG-dependent PKCs. Previous studies show that the ability of APC to inhibit β-catenin signaling is relatively insensitive to inhibition of GSK3 with Li^+. In keeping with its role in wg/wnt signaling the present study shows that APC2 activity, although not completely inhibited by Li^+, is more sensitive than APC. Most strikingly, a form of β-catenin (S37A), which is resistant to APC regulated ubiquitination and degradation, is as sensitive as wild-type β-catenin to APC2 regulation. The role of GSK3 in the regulation of β-catenin signaling activity is not clear. It was originally proposed that GSK3 directly phosphorylated a number of serine and threonine residues in the N-terminal of β-catenin although this has never been shown directly by phosphoamino acid analysis (50). GSK3 can also phosphorylate other members of the APC/axin complex and is now thought to mediate complex assembly rather than or as well as directly phosphorylating β-catenin (15). As pointed out previously the N-terminal stability regulating region of β-catenin contains a number of serine and threonine residues, only two of which are adjacent to a proline residue and conform to a modest GSK3 consensus (31). Importantly, serines 33 and 37 are present within a region (DSGIHS) with significant similarity to sequences known to be targets for the IκB kinase (IKK) family (49). Phosphorylation of the analogous residues in IκB by IKK leads to its association

with βTCRP which when complexed with skp and cullin targets the phosphorylated protein for ubiquitination (21). Because β-catenin is targeted for ubiquitination by the same complex these data suggest that, serines 33 and 37 in β-catenin, could also be phosphorylated by IKK, or a related kinase (14). Other serine and threonine residues in the N-terminal could be targets for GSK-3 or other kinases. Our demonstration that the S37A form of β-catenin is resistant to inhibition by APC but not APC2 together with their differential sensitivity to Li+ suggests that the two APC forms prepare β-catenin for phosphoryation by different kinases. Either route may be sufficient for the regulation of β-catenin signaling or both could be required.

It is important that the role of APC2 in actin associated events such as cell migration, and/or cell shape changes now be determined. APC and APC2 could cooperate in the cytoplasm or in association with microtubules and actin filaments respectively to control such processes as β-catenin signaling and cell motility as suggested by Barth et al (4). In addition interactions between microtubules and actin filaments occur during cell motility (47). The cellular location and many binding domains of APC2 suggest that it has multiple and perhaps dynamic functions.

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Footnotes

[1] Abbreviations: APC, adenomatous polyposis coli; APC2, APC-like gene; ARG, C. elegans APC related gene; bis, bisindoylmaleimide; DAG, diacylglceral; dAPC, Drosophila-APC; dAPC2, Drosophila APC2; EST, expressed sequence tag; hAPC2, human APC2; HDLG, human discs large protein; HGS/TIGR, Human Genome Sciences and The Institute for Genomic Research; mAPC2, mouse APC2; PAC, P-1 derived artificial chromosome; PJS, Peutz-Jeghers syndrome; RA, retinoic acid

Figure Legends

Table I: Expression of APC2

APC2 is expressed in many different tissues and cell lines including brain, breast, colon, and ovary at both the mRNA and protein level as observed by northern blotting, RT-PCR, and western blotting.

Figure 1: APC2 alignment, chromosomal localization and fine mapping

A) APC2 is 35% identical to human APC overall; however, the N-terminal dimerization domain is 68% identical. APC2 contains the conserved domain, the armadillo repeats, and five of the 20 amino acid repeats necessary for β-catenin binding and down regulation. APC2 lacks the three constitutive β-catenin binding sites and the DLG binding site. The SAMP repeats, necessary for axin binding, are poorly conserved in hAPC2. B) APC2 was mapped to chromosome 19p13.3 by FISH analysis using PAC clones identified through screening with a 1 kb cDNA fragment from the N-terminal region of APC2. Fine mapping using radiation hybrid screening by PCR located APC2 to the region on chromosome 19p13.3 containing markers WI-19632 and D19S883.

Figure 2: Expression of APC2 and western blot of cell lysates comparing APC2 and APC.

A.) Northern analysis of human multiple tissue and human cancer cell line poly(A)+RNA blots using a probe to the N-terminal region of APC2. APC2 expression is highest in the brain with varying levels in different regions. B.) SW480 and HBL-100 cell lysates were used to compare affinity purified APC2 rabbit antibody (5 μg/ml) to APC antibody-1 (1 μg/ml). No cross reactivity could be found. The truncated form of APC, T-APC, can be seen in SW480 cells and

full length APC, FL-APC, can be found in HBL-100 cells. APC2 is slightly smaller than FL-APC with possible splice variants or degradation products visible as well. Although both antibodies detected several immunoreactive species these did not coincide. MDA-MB-468 cell lysates were used to characterize the affinity purified IgY chicken antibody. C.) Varying protein patterns of APC2 are observed by western blot analysis of several different cell lysates using the IgY antibody (1 μg/ml). Equal amounts of protein (60 μg) were loaded in each lane.

Figure 3: Immunocytochemical staining for APC2.

A.) SKBR3 cells were stained using preimmune IgY (3 μg/ml). Little staining could be seen even in this overexposed image. B.) MDA-MB-157 cells stained using IgY antibody blocked with APC2 protein. C.) MDA-MB-157 cells stained for APC2 using APC2 IgY antibody (1ug/ml). APC2 can be seen conentrated along filamentous structures as well as concentrated along the membrane (arrows). D.) A549 cells were stained with APC2 affinity purified IgY antibody (1 μg/ml). The arrow indicates staining resembling the Golgi apparatus surrounding the nucleus. E.) A549 cells stained for APC2 as above. The box indicates a region of small vesicles/particles concentrated in a lamellipodial membrane. F.) A549 cells again stained for APC2. The arrows indicate staining resembling the Golgi apparatus surrounding the nucleus and staining along actin filaments.

Figure 4: APC2 localization at the Golgi apparatus and actin filaments.

A549 cells double stained with APC2 and PKCμ or phalloidin. Cells were treated with 2 μM cytochalasin D for 2hr. A1.) PKCμ clearly stains the Golgi apparatus. A2.) APC2 localizes to the Golgi apparatus. A3.) Double staining shows APC2 co-localization with PKCμ at the Golgi

apparatus. B1.) Phalloidin staining of actin filaments in A549 cells. B2.) APC2 is diffusely stained in the cytoplasm and appears to associate with actin filaments. B3.) Double staining shows APC2 associated with actin filaments. C1.) A549 cells treated with cytochalas in D and stained with phalloidin. Actin filaments are disrupted. C2.) Cells stained for APC2. APC2 staining in disrupted. C3.) Double staining shows that APC2 remains associated with actin filaments following treatment with cytochalasin D. D1-D3.) MDA-MB-157 cells treated with cytochalasin D and stained for actin and APC2 E1.) A549 cells stained for actin. The arrow indicates staining concentrated at cell-cell contact sites. E2.) Cells stained for β-catenin (1:100). The arrow indicates β-catenin in the same region as actin filaments. E3.) Double staining shows actin and β-catenin localized in the same region but rarely co-localized exactly. F1.) A549 cells treated with cytochalasin D and stained with phalloidin. F2.) Cells stained for β-catenin. The arrow indicates that β-catenin is disrupted similar to both actin and APC2. F3.) Double-staining shows that β-catenin remains associated with actin filaments.

Figure 5: APC2 association with actin filaments and β-catenin.

SKBR3 cells, both untreated and treated with 10^{-6} M RA, stained with either APC2, phalloidin, or β-catenin. A1.) Actin staining in untreated SKBR3 cells. The arrow indicates actin bundling at the membrane. A2.) Cells stained for APC2. The arrow indicates APC2 concentrated in the same region as actin. A3.) Double staining shows APC2 co-localization with actin filaments. In A1-A3 the microscope was focused on the cell surface, actin-containing structures. B1.) SKBR3 cells treated with RA and stained for actin. The actin filaments (arrow) are more organized in the treated cells. SKBR3 cells treated with RA are larger and more flattened. B2.) Cells stained for APC2. APC2 becomes much more concentrated along the membrane (arrow) and cell-cell

contact sites. B3.) Double staining shows APC2 at the edge of the cell with actin behind it. APC2 and actin are more co-localized at regions of cell-cell contact. C1.) SKBR3 cells stained for actin. C2.) Cells stained for β-catenin. SKBR3 cells have very little β-catenin and the staining is very diffuse. C3.) β-catenin and actin interaction is not apparent upon double staining. D1.) SKBR3 cells treated with RA and stained for actin. Again a more organized actin structure can be seen as indicated by the arrow. D2.) Upon treatment with RA, β-catenin becomes concentrated along the membrane and cell-cell contact sites as indicated by the arrow. D3.) Double staining indicates that after RA treatment, β-catenin and actin filaments co-localize along the membrane at cell-cell contact sites (indicated with the arrow). E1.) Distinct β-catenin staining along the membrane can be seen upon treatment with RA in SKBR3 cells (indicated with the arrowhead). F2.) APC2 staining also becomes more concentrated along the membrane after RA treatment (arrowhead). F3.) Double staining demonstrates that β-catenin and APC2 co-localize in regions along the membrane (arrowhead).

Figure 6: APC2 remains in the cytoplasmic fraction after RA treatment of SKBR3 cells. A.) A549 cells were fractionated into cytoplasmic, detergent soluble and insoluble fractions. APC2 was predominantly found in the cytoplasmic fraction. To determine the relative amount of APC2 in each fraction, equal cell equivalents of each fraction (i.e. 1/10 of each sample) were loaded in these experiments instead of equal amounts of protein. This blot was stripped and reprobed for β-catenin, which is found primarily in the detergent soluble membrane fraction. B.) APC2 is located in the cytoplasmic fraction of both RA treated and untreated SKBR3 cells. However, β-catenin levels not only increase but translocate to the detergent soluble membrane fraction after RA treatment. Untreated SKBR3 cells have very little β-catenin protein. Also note

that decreased mobility of three >200kD APC2 species in the detergent soluble phase following RA-treatment. In addition, RA-treatment decreases the intensity of two bands at ~51 K (arrowhead) in the cytoplasmic fraction.

Figure 7: APC2 regulation of β-catenin signaling.

APC2 inhibits β-catenin signaling in SW480 colon cancer cells. A) The ability of APC2 to inhibit β-catenin-regulated TOPflash activity is significantly reduced by bisindoylmaleimide (bis) but not by Calphostin C. In several experiments the effects of calphostin C ranged from 0 to 30% as shown here. Bisindoylmaleimide effects ranged from complete reversal of APC2 activity to 80% reversal as shown here. All transfections were done in triplicate and data plotted as percent inhibition. B) As shown previously, TOPflash activity induced by the S37A form of β-catenin is resistant to APC inhibition (11). Li^+ reduced the ability of APC to inhibit TOPflash acivity in SW480 cells by up to 35% (as shown here). In other experiments Li^+ was completely ineffective in inhibiting APC activity as shown previously (11). C) APC2 can inhibit TOPflash signaling induced by S37A β-catenin as well as wild-type β-catenin. Li+ consistently reduced the ability of APC2 to inhibit TOPflash activity by 50-75%.

Table I: APC2 Expression

Cell Line	Type	APC2 Expression	Cell Line	Type	APC2 Expression	Tissue	APC2 Expression
A1N4	Breast	$+^{2}$	SW480	Colon	$+^{1,2}$	Colon	$+^{2}$
BT20	Breast	$+^{1}$	LS-123	Colon	$+^{1}$	Ovary	$+^{2}$
HBL-100	Breast	$+^{1,2}$	T84	Colon	$+^{1}$	Testis	$+^{2}$
MCF-7	Breast	$+^{2}$	A549	Lung	$+^{1,2}$	Spleen	$-^{2}$
MCF-10A	Breast	$+^{2}$	Calu-3	Lung	$+^{1}$	Periph. Bl. Luek.	$-^{2}$
MDA-MB-134	Breast	$+^{2}$	G361	Melanoma	$+^{2}$	Brain	$+^{2}$
MDA-MB-453	Breast	$+^{1,2}$	SKOV3	Ovary	$+^{1}$	Sm. Intestine	$+^{2}$
MDA-MB-231	Breast	$+^{2}$	U87	Brain	$+^{1}$	Prostate	$+^{2}$
MDA-MB-468	Breast	$+^{1,2}$	A-431	Epidermoid	$+^{2}$	Thymus	$-^{2}$
MDA-MB-435	Breast	$+^{1}$	MOLT-4	Leukemia	$-^{2}$		
SKBR3	Breast	$+^{1,2}$	Burkitt's Raji	Lymphoma	$-^{2}$		
T47D	Breast	$+^{1,2}$	HL-60	Leukemia	$-^{1}$		
ZR-75-1	Breast	$+^{1}$	K-562	Leukemia	$+^{1}$		

1 = protein, 2 = RNA

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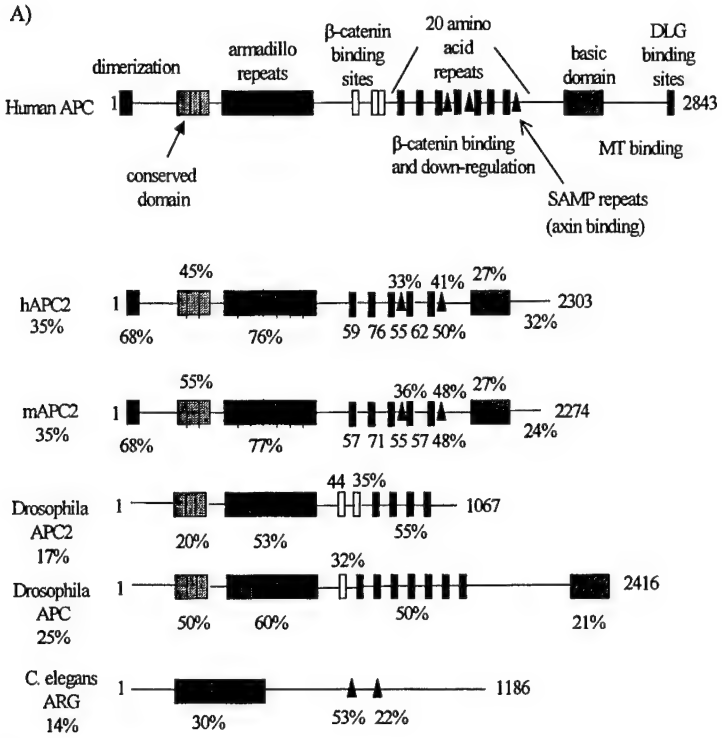

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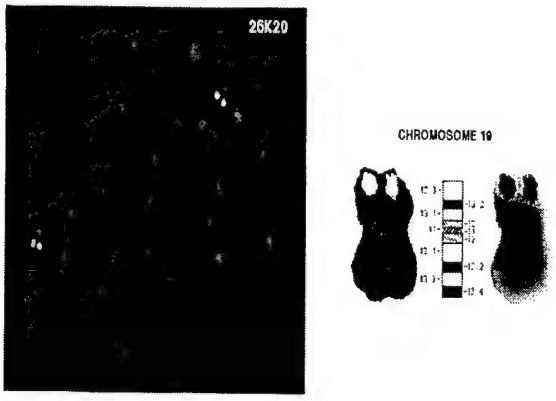


Chromosome 19p13.3

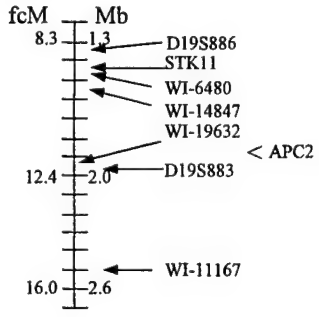
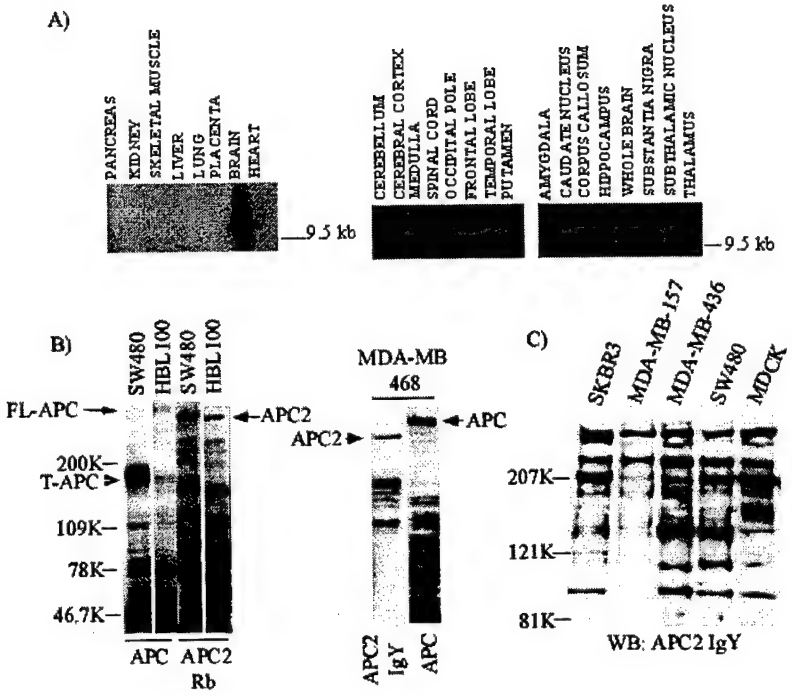


FIGURE 1

FIGURE 2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APC
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APC
C)
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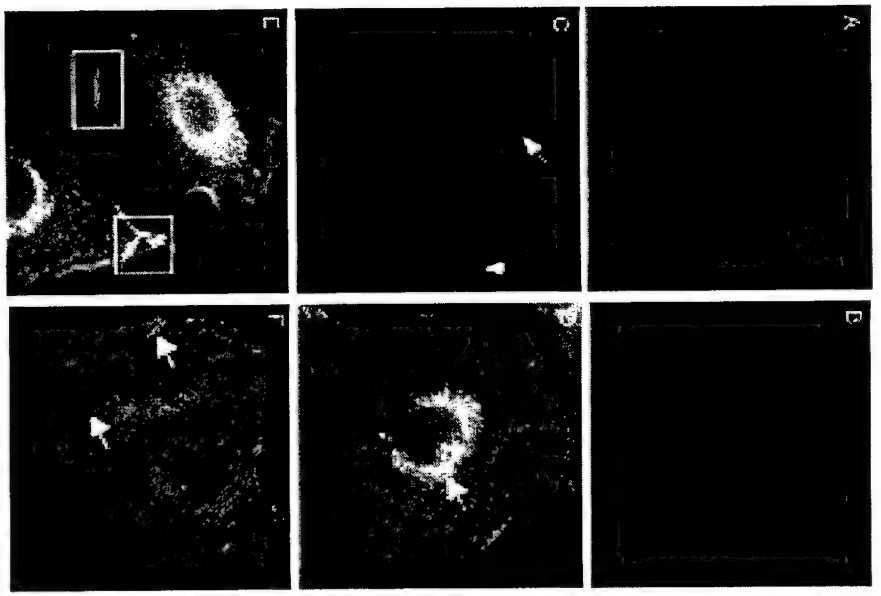
FIGURE 3



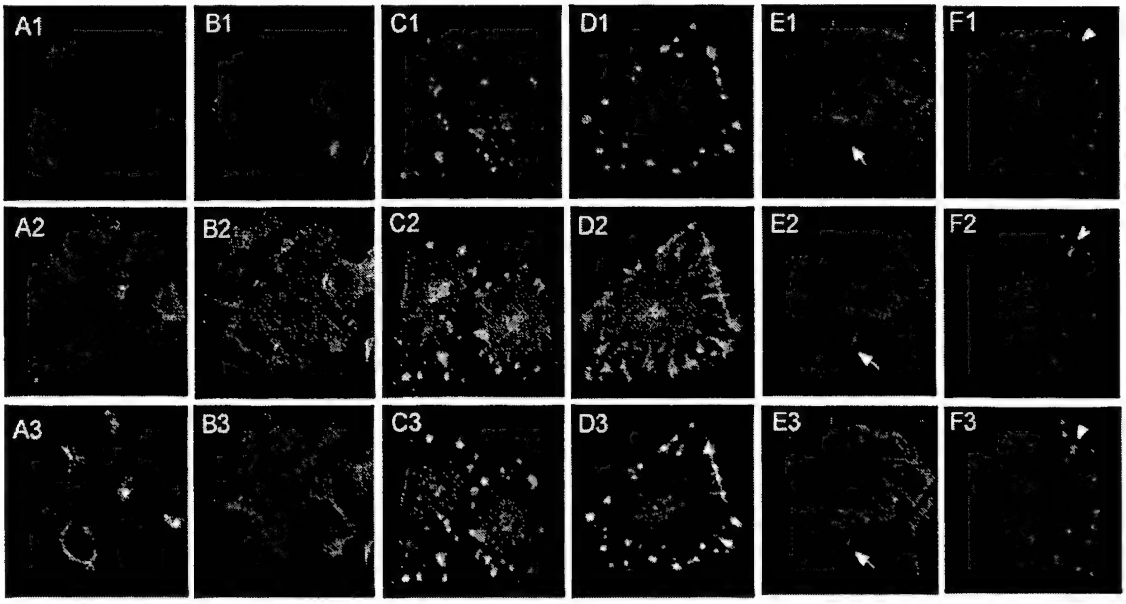


FIGURE 4

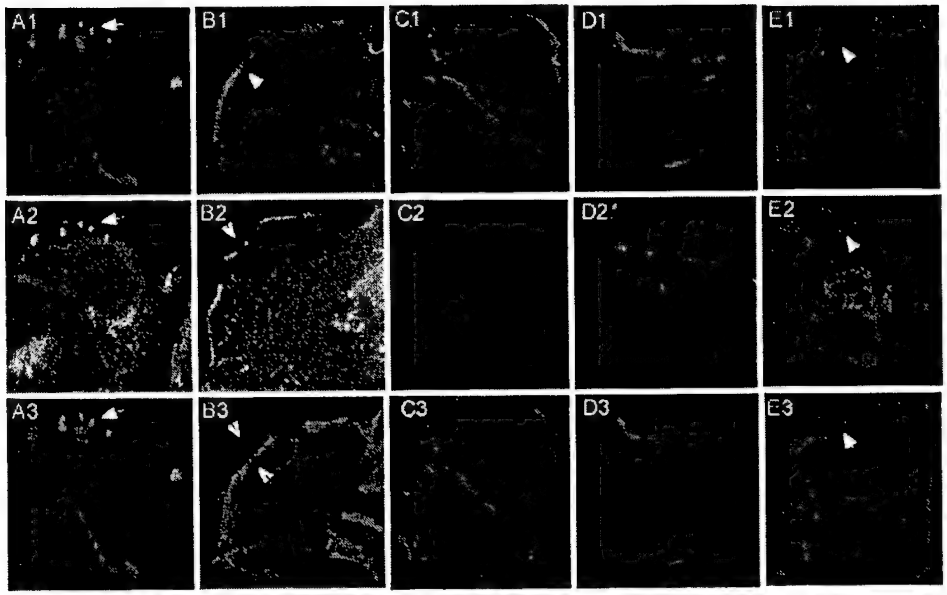


FIGURE 5

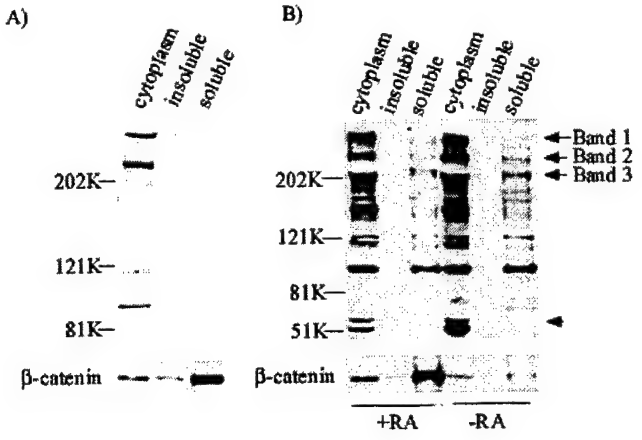

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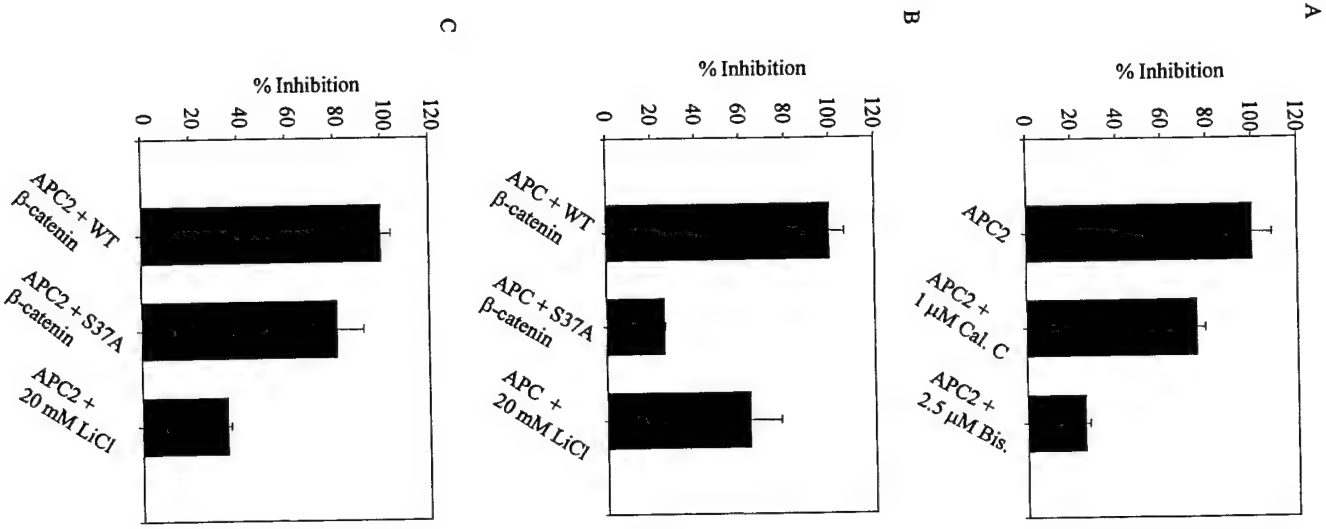
FIGURE 6

FIGURE 7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APC + S37A β-catenin
APC + 20 mM LiCl
C
% Inhibition
APC2 + WT β-catenin
APC2 + S37A β-catenin
APC2 + 20 mM LiCl

The Role of Cadherin, β-catenin, and AP-1 in Retinoid-regulated Breast Cancer Cell Differentiation and Proliferation

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Running Title: Cadherin Expression Mimics Retinoid Effects

Keywords: Cadherins, β-catenin, Retinoic Acid, AP-1, LEF

Abstract

Vitamin A derivatives (retinoids) are potent regulators of cell proliferation and epithelial cell differentiation. Retinoids inhibit the function of the proto-oncogene transcription factor complex, AP-1, and also stabilize components of the adherens junction, a tumor suppressor complex. When treated with 9-*cis* retinoic acid the breast cancer cell line, SKBR-3, undergoes a dramatic change in cell morphology, an increase in cell-cell adhesion strength, and an increase in membrane associated β-catenin. The present work demonstrates that in SKBR-3 cells, retinoic acid increases cadherin expression, decreases cytoplasmic β-catenin levels and decreases AP-1 and LEF-reporter activity. However, neither expression of a dominant negative c-jun, nor expression of β-catenin promoted epithelial differentiation. Consistent with this exogenous expression of c-jun did not reverse the effects of retinoic acid. In contrast, exogenous E-cadherin expression completely mimicked the effects of retinoic acid on epithelial differentiation and reduced cytoplasmic β-catenin levels. Growing cells in low calcium-containing medium to inhibit cadherin function blocked the morphological effects of retinoic acid and prevented the retinoid-induced decrease in cytoplasmic β-catenin. However this treatment did not prevent the retinoic acid affects on LEF-reporter activity. Similarly, exogenous expression of E-cadherin did not mimic the inhibitory effects of RA on cell proliferation. These data show for the first time that the effects of retinoic acid on epithelial differentiation can be separated from its effects on cell proliferation.

Introduction

Retinoids are important regulators of cell proliferation and epithelial differentiation, and can act as potent antitumor agents. For example, retinoic acid (RA) inhibits the formation of papillomas in the skin of mice and can prevent the transformation of mouse JB6 cells {1526, 1556,1557}. Retinoids also inhibit the growth of several human cancers, including melanoma, as well as colon and prostate cancer {1560, 1561,1562, 1563, 1564}. Retinoids are potent differentiating agents and can induce differentiation of endothelial, melanoma, neuroblastoma, and lung cancer cells {1727}. Retinoids also inhibit growth and induce differentiation of breast cancer cells. For example, Anzano, *et al* showed that 9-*cis*-RA reduced tumor incidence, average number of tumors and average tumor burden in a rat breast cancer model {1155}. Retinoids also inhibit the growth of a number of breast cancer cell lines {1558, 1208}. In some instances the effects of RA on cell growth have been attributed to the ability of RA to down-regulate AP-1 activity ($$). However, it is not clear if the effects of retinoic acid on epithelial differentiation can be separated from its effects on cell growth. We and others have previously demonstrated that the effects of retinoids may involve modulation of adherens junction structure and function {1401, 1565, 1523}.

The adherens junction is a molecular complex that is essential for initiating and maintaining strong cell-cell adhesion in epithelial cells {144}. The basic components of the adherens junction include a transmembrane cadherin molecule, the cytoplasmic catenins, and the actin cytoskeleton {1129}. Cadherins are calcium-dependent cell adhesion molecules that are involved in the organization of the developing embryo, and are essential for the maintenance of tissue integrity in the adult {81}. In epithelial cells, loss of function or expression of E-cadherin is well correlated with the progression of tumors to a more invasive phenotype {1540}. Moreover, expression of other cadherins has proven to be sufficient to inhibit the invasive phenotype and thus compensate for a loss of E-cadherin {1755, 1775, 1554}. Loss of cadherin function may also be mediated in other ways. For

example, loss of α- or β-catenin protein expression can disrupt normal cell-cell adhesion {1776, 1777}. Alternatively, cadherin function can be modulated by tyrosine kinase activity as was first demonstrated in v-src transfectants {1316, 1375}. Expression of the v-*src* oncoprotein, which is known to transform cells, phosphorylates tyrosine residues on β-catenin and the cadherins and disrupts the adherens junction, resulting in a shift to a fibroblastoid phenotype that exhibits increased invasiveness {714, 1316}. Finally, loss of cadherin function may be mediated by activity of the transcription factor complex, AP-1. AP-1 is made up of the proto-oncogenes jun and fos, and its activity is associated with cell proliferation and neoplastic transformation {1733}. Fialka, *et al* showed that activation of c-jun in mammary epithelial cells resulted in a loss of epithelial polarity, a disruption of intercellular junctions and normal barrier function, and the formation of irregular multilayers. This was accompanied by a reduction in the association between E-cadherin and β–catenin in a manner that is independent of tyrosine phosphorylation {1523}.

Previously we have shown that retinoids have a profound effect on cell-cell adhesion {1401}. In the breast cancer cell line, SKBR-3, RA induces a dramatic epithelialization that is accompanied by an increase in cell-cell adhesion strength and an increase in the amount and triton insolubility of β-catenin protein. We now show that in SKBR-3 cells RA induces the expression of a cadherin, and inhibits AP-1-reporter activity and β-catenin signaling. Furthermore, our work demonstrates that cadherin expression and function are necessary and sufficient to mediate the effects of RA on adhesion and epithelial differentiation but are not required for RA-mediated inhibition of β-catenin/ LEF signaling activity or cell proliferation. In contrast, neither exogenous expression of β-catenin or c-jun nor inhibition of AP-1 alters the effects of RA on epithelial differentiation.

Materials and Methods

Cell lines and treatments

SKBR-3 cells were obtained from ATCC and grown in Dulbelco's modified eagle's medium (Gibco) plus 5% fetal bovine serum as described previously {310}. Cells were treated with 1 μM 9-*cis* retinoic acid or ethanol for 48 hours. TPA (Sigma) was used at 100 nM overnight.

Antibodies

An anti-β-catenin monoclonal antibody (Transduction Labs) was used for immunocytochemistry (1:100) and western blotting (1:500). An anti-β-catenin polyclonal antibody, SHB7 (Kindly provided by David Rimm, Yale University, New Haven, CT) was used for immunocytochemistry (1:500) and immunoprecipitation. An anti-α-catenin polyclonal antibody, YR4 (Kindly provided by David Rimm, Yale University, New Haven, CT) was used for immunoprecipitation. An anti-E-cadherin monoclonal antibody (Transduction Labs) was used for immunocytochemistry (1:100). A pan-cadherin polyclonal antibody (Sigma) was used for immunocytochemistry (1:100) and western blotting (1:500). An anti-flag monoclonal antibody (Kodak) was used for immunocytochemistry (1:400) and western blotting (1:500). An anti-c-jun polyclonal antibody (Sigma) was used for western blotting (1:500). Secondary antibodies, goat-anti rabbit and goat-anti mouse both peroxidase-labeled (Kirkegaard and Perry) and FITC-labeled (Kirkegaard and Perry) or Texas Red-conjugated (Jackson Immunochemicals) were used at 1:100. All antibodies were diluted in 5% skim milk in Phosphate-buffered saline (PBS) for western blotting or 6% normal goat serum for immunocytochemistry (ICC).

Vectors

Vectors encoding either wild type or a degradation-resistant mutant of β-catenin (both HA and Flag-tagged) were kindly provided by Keith Orford, Georgetown University, Washington, D.C. A wild type human E-cadherin vector was kindly provided by Carien Nesson, Memorial Sloan-Kettering Cancer Center, New York, N.Y. A dominant-negative c-jun construct as well as wild type and mutant AP-1 luciferase constructs were kindly provided by Powell Brown, University of Texas Health Science Center at San Antonio, San Antonio, TX. A human c-jun expression vector was kindly provided by Bart van der Burg, Hubrecht Laboratory, The Netherlands. A GFP expression vector was used (pEGFP, Clontech). An LEF-luciferase reporter construct, pTOPFLASH was provided by Marc Van de Wettering {1508}.

Cellular Subfractionation

Cells from confluent 10 cm dishes were isolated and dounce homogenized in a hypotonic solution (10 mM Tris, 0.2 mM $MgCl_2$, pH 7.5). The homogenate was spun first for 10 min. at 3000 X g, to remove nuclei. The supernatant was then ultracentrifuged at 150,000 X g for 1 hour. The supernatant, defined as the cytoplasmic fraction, was added to 4 volumes of ethanol and the proteins precipitated overnight. The proteins were then reisolated by ultracentrifugation and solubalized in sample buffer (2% sodium dodecyl sulfate (SDS), 60 mM Tris, pH 6.8, 10% glycerol). The pellet from the original ultracentrifugation was solubilized in a 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0) for 30 min., then reclarified in a microcentrifuge for 15 min. to remove insoluble material and cell debris. The resulting supernatant, the NP-40 soluble fraction, was then added to sample buffer.

Immunoprecipitations

A confluent 10 cm dish was lysed in a 1% Nonidet P-40 buffer. The lysate was then clarified in a microcentrifuge and the supernatant was precleared with 1 mg of normal rabbit serum and 100 µl of protein-A agarose (Boerhinger Manheim). The lysate was then immunoprecipitated overnight with the appropriate antibody. The bound proteins were isolated with protein-A agarose beads (Boerhinger Manheim), and the beads were washed 6 times. The beads were boiled in Laemlli buffer {1030} with β-mercaptoethanol and western blot was performed as described below.

Western Blotting

Protein content in the samples was measured by the Bio-Rad D_C Protein Assay. 25 µg of protein were separated on an 8% reducing polyacrylamide minigel (Novex), transferred onto nitrocellulose (Protran) and blocked overnight in 5% skim milk. The blot was then probed with an appropriate antibody, followed by a secondary peroxidase-labeled antibody (Kirkegard and Perry), and the bands visualized by Enhanced chemiluminescence (Amersham). The blots were then stripped at 50°C for 30 min. (stripping solution: 62.5 mM Tris, pH 7.5, 2% SDS, 1.7% (v/v) β-mercaptoethanol), washed 2 X in PBS, and blocked in 5% milk prior to reprobing.

Immunocytochemistry

Cells were grown on 12 mm coverslips. Most coverslips were fixed in 100% ice cold methanol for 3 min. at -20°C. In order to retain green fluorescent protein staining, cells were fixed in 2% paraformaldehyde for 30 min. followed by 0.5% Triton-X 100 for 5 min. Coverslips were then blocked in 3% ovalbumin for 30 min. For double staining, the first primary antibody was bound

overnight at 4°C, followed by a FITC-conjugated secondary antibody for 1 hour at RT. Next, the second primary antibody was bound at RT for 1 hour, followed by a Texas Red-conjugated pre-absorbed secondary antibody for 1 hour at RT. Cells were mounted (Vectashield) and visualized on a Zeiss microscope. There was no crossover between fluorescein and Texas Red channels.

For pan-cadherin staining antigen retrieval was performed. Following fixation, coverslips were placed in 1 liter of 0.01 M citrate buffer, pH 6.0 (18 ml of 0.1 M citric acid, 82 ml 0.1 M sodium citrate pH 6.0, 900 ml dH_2O). The coverslips were microwaved for 30 min. on high, then allowed to cool slowly before blocking.

GFP sorting and immunocytochemistry of transfected cells

1 X 10^6 cells were plated in a 10 cm dish. The cells were transfected with 10 μg of a green fluorescent protein expression vector, pEGFP (Clontech) and 50 μg of the plasmid of interest. Cells were transfected for 6 hours by the calcium phosphate method {1131}, then shocked with media containing 20% glycerol for 4 min. Cells were then washed 3 X in PBS and incubated for 24 hours. Cells were then trypsinized and sorted by FACS, isolating cells expressing high levels of GFP. Cells were replated on 12 mm coverslips, then treated with RA or vehicle for 48 hours and fixed and stained as described above.

Luciferase reporter assays

Cells were seeded in 12 well plates at 1 x 10^5 cells per well. Cells were transiently transfected with 1 μg of the either the wild type or mutant AP-1 luciferase construct or with the LEF-reporter pTOPFLASH {1508}; with 0.02 μg of pCMV-Renilla Luciferase (Promega); and with 5 μg WT β-catenin {1530} (LEF-reporter assays only). The calcium phosphate method was used {1131}. RA

treatment was initiated 24 hours post-transfection. Luciferase activity was monitored using the DUAL-Luciferase Assay System (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency by comparison with the constitutively expressed Renilla Luciferase.

Results

Retinoic acid increases endogenous cadherin expression in SKBR3 cells

Previously we have shown that retinoids have a profound effect on cell-cell adhesion and cell proliferation {1155, 1401}. In the breast cancer cell line, SKBR-3 RA induces a dramatic epithelialization that is accompanied by an increase in cell-cell adhesion strength, decreased cell proliferation, and an increase in the expression and triton insolubility of β-catenin {1401}. The increase in β-catenin expression is due to an increase in protein stability and not in steady-state RNA levels {1401}. RA-induced effects on cell morphology and the movement of β-catenin to points of cell-cell contact are dependent upon high levels of extracellular calcium (>50 uM) suggesting a role for cadherin-mediated adhesion {1129, 1401}. However, SKBR-3 cells have a homozygous deletion of the E-cadherin gene and it was not clear what molecule was recruiting β-catenin to the membrane following RA treatment {1320}. In order to examine cadherin expression in SKBR-3 cells, immunocytochemistry and western blot were performed using a pan-cadherin polyclonal antibody (Sigma) that was raised against the highly conserved C-terminal tail of chicken N-cadherin. Figure 1 (A, B) reveals that RA dramatically increases the expression of a cadherin at points of cell-cell contact, a staining pattern that closely parallels that seen previously for β-catenin {1401}. It should be noted that, to successfully examine the cadherin by immunocytochemistry, cells were microwaved following fixation. This procedure dissociates protein-protein interactions while not affecting their localization. Microwaving enabled the pan-cadherin antibody to bind to a site that was previously unavailable, presumably due to interference by bound β-catenin.

Next, western blot analysis revealed that cadherin expression is increased in the membrane-associated fraction in the presence of RA (Figure 1C). β-catenin levels increased markedly in the

detergent soluble and insoluble membrane fractions but not in the cytoplasmic fraction (Figure 1D). Although total alpha catenin levels did not increase markedly following RA-treatment there was a large increase in the proportion of α-catenin present in the detergent soluble fraction following RA treatment (Figure 1E). Unlike β-catenin, a significant amount of α-catenin was present in the cytoplasmic fraction before and after RA-treatment. Although the RA-induced cadherin could not be identified (see discussion) it does clearly associate with α-catenin and β-catenin. Immunoprecipitation of α-catenin (Figure 1F), and β-catenin (Figure 1G) co-immunoprecipitate the cadherin, as revealed by western blotting with the pan-cadherin antibody. These data indicate that the effects of RA on adherens junction function might be mediated by, increased expression and/or function of cadherin, β-catenin or both. Other studies have shown that activation of c-jun/AP-1 can inhibit adherens junction function and β-catenin/cadherin interactions ($$). Because one notable action of RA is the inhibition of AP-1 activity we next investigated the role of AP-1 in the differentiation promoting actions of RA{1733, 1525}.

Inhibition of AP-1 activity is neither necessary nor sufficient to mimic the effects of RA on epithelial differentiation

Chen, *et al* showed that AP-1 activity is relatively high in SKBR-3 cells {1524}. To determine the effects of RA on AP-1 activity in SKBR-3 cells, reporter assays were performed using an AP-1 reporter construct consisting of the collagenase promoter region (-74 to +63) upstream of a luciferase reporter gene. A mutant construct with a three nucleotide mutation in the AP-1 site, was used as a negative control. As expected, RA reduced AP-1 reporter activity in SKBR-3 cells in a dose dependent

manner (Figure 2A). A dominant-negative c-jun, TAM-67 also significantly reduces AP-1 reporter activity (Figure 2A).

To determine whether AP-1 function is necessary and sufficient to inhibit the effects of RA on epithelial differentiation, SKBR-3 cells were transfected with TAM-67 or with a c-jun expression vector (kindly donated by Bart van der Burg, Hubrecht Laboratory, The Netherlands) {1789}. Neither TAM-67 nor c-jun altered cadherin or β-catenin proteins levels in cell lysates (Figure 2B-D). TAM-67 does not induce any changes in cell morphology, β-catenin expression, or cadherin expression detected by immunocytochemistry (Figure 3A-D). Likewise, transfection of c-jun is not sufficient to inhibit the function of RA in altering cell morphology and cadherin or β-catenin expression (Figure 3E-L).

β-catenin expression is not sufficient to mimic the effects of RA on epithelial differentiation

Because the most significant molecular change observed after RA-treatment is increased β-catenin protein levels we next wanted to determine whether overexpression of β-catenin was sufficient to mimic the effects of RA. Immunocytochemistry was performed on SKBR-3 cells transiently transfected with β-catenin. SKBR-3 cells express very little β-catenin and have a rounded morphology {1401}. SKBR-3 cells transfected with vector alone have a similar phenotype (Figure 4A). In wild type β-catenin transfected cells, the majority of the exogenous β-catenin could be found in the cytoplasm, and nucleus (Figure 4C, also see {1787}). However, despite the high level of β-catenin expression, there was no morphologic change (Figure 4C), nor any increase in the expression of the cadherin (Figure 4D). In contrast, RA-treatment resulted in a dramatic morphologic change and localization of β-catenin to sites of cell-cell contact along the membrane (Figure 4B and 1B). Cells were also transfected with a mutant form of β-catenin that is more resistant to degradation {1530}.

Results with this mutant, S37A β-catenin were similar to those with the wild type vector (Figure 4D, E). Western blots of wild type β-catenin transfected cells confirmed that cadherin levels did not change (Figure 4G). The increase in β-catenin appears modest because only 10-20% of the cells are transfected and because transiently transfected β-catenin accumulates predominantly in the cytoplasm, and these samples represent whole cell protein levels.

Cadherin expression is sufficient to mimic the effects of RA on epithelial differentiation

RA-induced changes in SKBR3 cell morphology are reversed by growing cells in reduced calcium (50 uM), suggesting that the calcium dependent adhesive function of a cadherin is necessary to mediate the effects of RA {1401}. 50 uM Ca++ is sufficient to alter cadherin function but does not change intracellular calcium levels. To determine whether expression of a cadherin is sufficient to mimic the RA-induced effects, SKBR-3 cells were transfected with an E-cadherin construct, hecD pCDNA3 (Kindly donated by Carien Nesson, Sloan-Kettering Memorial Cancer Center, New York, NY). The cells were co-transfected with a Green Fluorescent Protein expression vector (pEGFP) and green cells were separated by FACS, replated, and grown with or without RA for 48 hours. As expected, in control transfected cells the effects of RA on cell morphology were dramatic (Figure 5B) compared to untreated cells (Figure 5A). Expression of E-cadherin is sufficient to mimic the RA-induced changes in cell morphology (Figure 5C), whereas expression of β-catenin is not (Figure 5D).

E-cadherin expression also increases the expression of β-catenin at the membrane and other sites of E-cadherin expression as revealed by immunocytochemistry (Figure 5 E, F). Transfection with the empty vector (pcDNA3) failed to increase β-catenin expression levels (Figure 5G, H). Western

* Diff. of medium $[Ca^{++}]$

blot analysis of whole cell lysates of cells transiently transfected with the full length E-cadherin constructs revealed that expression of E-cadherin resulted in increased β-catenin protein levels (Figure 5I). Note that the increase in β-catenin expression is less than that induced by RA because only 10-20% of cells are transfected with the E-cadherin gene. Thus, these results strongly implicate the expression of a cadherin as one mechanism by which RA acts to induce epithelial cell differentiation.

Cadherin expression and function is neither necessary nor sufficient to mediate the RA effects on SKBR3 cell proliferation

It is well known that in RA-sensitive cells, treatment with RA decreases cell proliferation within 24 hours ($$). We showed previously that, DNA-synthesis in SKBR3 cells, is markedly inhibited by RA ($$). E-cadherin has also been implicated as a tumor suppressor, although there is little evidence that it directly affects cell proliferation ($$). We next wanted to investigate if the short-term effects of RA on DNA synthesis could be mimicked by transfection of E-cadherin. Figure 6A shows that in control cells 48 hours after RA treatment, DNA synthesis, as measured by tritiated thymidine uptake, was reduced by 50%. E-cadherin transfection alone did not reduce DNA synthesis in this time period but, as expected, did exert a marked morphological transformation (see Figure 5). DNA-synthesis in E-cadherin-transfected cells was still reduced by RA. In this experiment E-cadherin-transfected cells sorted by FACS were compared with non-expressing cells. These experiments show that cadherin expression is not sufficient to mediate the growth inhibitory effects of RA. However, it is possible that calcium-dependent adhesion is still required for RA to inhibit cell proliferation. To test this we grew cells in 2 mM calcium and in 50 uM calcium and compared the effects of RA. RA-treatment inhibited tritiated thymidine uptake under both conditions even though the morphological effects of RA were completely reversed in cells growing in 50 uM calcium (Figure

6B). This result shows that calcium-dependent adhesion is not required for RA to inhibit cell proliferation

Cadherin function is necessary for RA treatment to reduce the cytoplasmic pool of β-catenin

The mechanism whereby RA increases the membrane pool of β-catenin is most likely the result of increased cadherin expression. Several studies have shown that exogenous expression of cadherins can recruit cytoplasmic β-catenin to the membrane pool and reduce β-catenin/LEF signaling, effects that depend upon the ability of the cadherin cytoplasmic tail to bind β-catenin ($$). We next wanted to test if RA-treatment could reduce cytoplasmic levels of exogenously expressed β-catenin in SKBR3 cells. Figure 7A shows that, cytoplasmic levels of β-catenin are markedly elevated following transient transfection. RA-treatment reduces the level of cytoplasmic β-catenin significantly. RA also decreased the cytoplasmic level of the constitutively stable S37A form of β-catenin. If the ability of RA to reduce cytoplasmic β-catenin depended upon cadherin function we would expect that treatment of cells with low calcium medium should reverse these effects of RA. Figure 7B shows that exposure of cells to low calcium medium prevents the RA-mediated decrease in the level of cytoplasmic β-catenin in transiently transfected cells. These results strongly suggest that the ability of RA to reduce cytoplasmic β-catenin is solely due to increased cadherin expression and is not a result of degradation of the cytoplasmic pool. Taken together these data show that the effects of RA on mediating epithelial cell differentiation, reducing cytoplasmic β-catenin and recruiting it to the membrane are the result of a RA-induced increase in the expression and/or function of a cadherin.

Calcium-dependent cell-cell adhesion is not required for RA to decrease β-catenin/LEF signaling

The ability of RA-treatment to reduce cytoplasmic β-catenin suggested that it might also inhibit β-catenin/LEF signaling in SKBR3 cells in a cadherin-dependent fashion. However, although RA did significantly decrease β-catenin/LEF reporter activity, it was equally effective in cells growing in reduced calcium medium in which cytoplasmic β-catenin levels were unaffected by RA (Figure 7C). These data indicate that the ability of RA to interfere with β-catenin/LEF activity and to reduce cell proliferation is independent of its effects on adherens junction function and differentiation.

Discussion

Retinoids are potent regulators of cell proliferation and epithelial cell differentiation. The actions of RA are mediated through retinoid receptors, which upon ligand binding affect three important transcriptional programs. RA-activated RAR/RXR heterodimers bind to the RARE-containing promoter elements of many genes to displace co-repressors and activate transcription ($$). RA-activated RARs can also inhibit the action of the fos/jun AP-1 complex thereby blocking the transcription of AP-1 regulated genes ($$). Finally, we recently demonstrated that RA-activated RAR but not RXR can inhibit β-catenin/TCF transactivation by directly binding to β-catenin ($$). The pleiotropic actions of RA are probably a result of the differential regulation of these three pathways. However, it is not clear which of these pathways accounts for the effects of RA on epithelial cell differentiation and proliferation. Using the breast cancer cell SKBR3 as a model system we now show that these two actions of RA can be separated.

RA-effects on SKBR3 cell epithelial differentiation are not regulated by AP-1 or β-catenin/TCF pathways but do require calcium-dependent cell adhesion and cadherin function

In earlier studies we showed that RA-treatment of SKBR3 cells induces a dramatic morphological change, increases cell-cell adhesion, increases the half-life and protein levels of β-catenin and decreases cell proliferation{1401}. Using receptor specific ligands we showed that these changes were most likely mediated by RAR/RXR heterodimer activation and were cell-autonomous. In the present study we have used dominant negative c-jun as well as overexpression of c-jun to demonstrate that regulation of AP-1 does not mediate the differentiation-promoting action of RA.

Similarly, overexpression of β-catenin did not mimic or inhibit RA effects on differentiation. In contrast exogenous expression of E-cadherin could mimic RA-effects on morphology and β-catenin levels and recruitment to the membrane. Consistent with this growing cells in reduced calcium, to block cadherin function, reversed the effects of RA. These observations suggest that RA-treatment of SKBR-3 cells might increase cadherin expression or function in an AP-1 and β-catenin/TCF-independent manner. However, SKBR3 cells have a homozygous deletion of the E-cadherin gene ($$). In the present study we used a polyclonal pan-cadherin antibody raised against a region of the highly conserved C-terminal, to investigate RA-regulation of cadherin expression. This antibody recognizes most cadherins except, notably E-cadherin {1552, 1756}. RA dramatically increased levels of a cadherin recognized by this antibody, the majority of which was found at cell-cell contact sites. Immunoprecipitation studies confirmed that the SKBR3 cadherin could form a complex with both α-catenin and β-catenin. A variety of approaches have shown that the RA-induced cadherin is not E-, N-, P-, or LI-cadherin, cadherin-6 or-11 (results not shown). However, our attempts to identify this cadherin by classical methods have proven unsuccessful. This suggests that the unidentified cadherin may be a novel cadherin that is regulated directly or indirectly by RA. Whatever the identity of the cadherin is, its expression and function is necessary and sufficient the mediate the morphological effects of RA on SKBR3 cells.

Cadherin expression and function is not necessary to mediate the effects of RA on SKBR3 cell proliferation

In other studies we showed that SKBR3 cells have quite high constitutive AP-1 activity and that the same dominant negative c-jun we used in the present study inhibited cell proliferation {1789}. If, as

we expected, the effects of RA on cell proliferation were mediated specifically by inhibition of AP-1 we hypothesized that cadherin expression and function would not be directly involved in the growth inhibitory effects of RA. Consistent with this idea, neither exogenous expression of E-cadherin, nor growing cells in reduced calcium medium, affected DNA synthesis or the ability of RA to inhibit DNA synthesis.

Cadherin expression and function is required for RA-treatment to reduce cytoplasmic β-catenin levels but is not required for RA-inhibition of β-catenin/TCF signaling

In contrast to their high AP-1 activity SKBR3 cells have very low levels of β-catenin and TCF-reporter activity. Consequently, it is unlikely that over-activation of this pathway is involved in the transformed phenotype of these cells as it is in colon cancer ($$). However, the ability of RA to dramatically increase membrane β-catenin levels without increasing cytoplasmic β-catenin suggested to us that the RA-induced cadherin might act to recruit excess β-catenin to the membrane. For example, it is well known that exogenous cadherin expression can sequester β-catenin from the cytoplasmic pool and reduce β-catenin/TCF reporter gene activity ($$). To test this we transfected cells with wild-type and a stable mutant form of β-catenin and treated the cells with RA. As expected, cytoplasmic β-catenin levels were elevated in the transfected cells. RA-treatment resulted in a marked reduction of cytoplasmic β-catenin and an increase in membrane-associated β-catenin. Reduced calcium medium completely inhibited the ability of RA to reduce cytoplasmic β-catenin demonstrating that cadherin function was required. However, RA-treatment inhibited β-catenin/TCF reporter activity independently of cadherin function and reduction of cytoplasmic β-catenin. This result is consistent

with our recent demonstration that RA-activated RAR is able to bind directly to β-catenin and inhibit β-catenin/TCF signaling ($$).

Taken together these results suggest the following model (Figure 8). In SKBR3 cells, RA increases the expression of a β-catenin binding cadherin that mediates strong cell-cell adhesion and which is sufficient to mediate the effects of RA on morphology and recruitment of β-catenin to the cell membrane. Since these effects are not regulated by AP-1 or β-catenin/TCF signaling it is likely that they are mediated via activation of RARE-containing genes. However, this pathway is not required for RA-mediated growth inhibition. Since β-catenin/TCF signaling is very low in SKBR3 cells these data are consistent with a role for RA-RAR inhibition of AP-1 activity in mediating the RA-effects on SKBR3 cell proliferation ($$). In other cells such as colon cancer cells that do have dysregulated β-catenin/TCF signaling RA could inhibit cell proliferation by directly interfering with this pathway rather than AP-1 ($$). Thus, the remarkably broad effects of RA on the growth and differentiation of many different epithelial cancers may well be explained by its ability to differentially regulate the activity of these three important pathways.

References

Acknowledgements

Figure 1: RA induces the expression of a cadherin. A, B. SK-BR-3 cells were fixed in methanol and microwaved. A pan-cadherin antibody was used to detect cadherin expression in RA untreated (A) and RA treated (B) cells. RA dramatically increases the expression of a cadherin at sites of cell-cell contact (B). C. Cellular subfractionation confirms that RA increases cadherin expression in the membrane-associated pool. Both β-catenin and α-catenin levels are also increased in the membrane pool following RA-treatment (D, E). Immunoprecipitates of α-catenin and β-catenin contain cadherin following RA-treatment (F, G)

Figure 2: A. RA and dominant negative c-jun inhibit AP-1 reporter activity. RA and a dominant-negative c-jun, TAM-67 reduce the level of AP-1 reporter activity. SK-BR-3 cells were transfected with an AP-1 responsive luciferase reporter construct and treated with either 100 nM TPA or with the indicated concentrations of RA. A mutant AP-1 reporter was used as a negative control. Results are plotted as the percent of reporter activity compared with the wild type construct in untreated cells. As shown previously, RA reduces the amount of AP-1 activity {1525}. Co-transfection with the TAM-67 dominant-negative c-jun reduced AP-1 reporter activity to background levels. B-D. AP-1 activity does not affect the expression of β-catenin or the endogenous cadherin. SK-BR-3 cells were transfected with Flag-tagged Tam-67 or with c-jun and grown +/- RA for 48 hours. Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. AP-1 activity had no effect on cadherin or β-catenin expression (B) despite the high levels of c-jun (C) and TAM-67 expression (D). Note that the endogenous c-jun is detected in the control cells and that cells transiently transfected with c-jun did express detectably more c-jun even though less than 20% of the cells are transfected.

Figure 3. Inhibition of AP-1 activity is neither necessary nor sufficient to mimic the effects of RA on cell morphology and cadherin and β-catenin expression. A-D. SK-BR-3 cells were transfected with the flag-tagged construct, TAM-67, then stained with an anti-flag antibody (A, C) or with a β-catenin antibody (B) or a pan-cadherin antibody (D). Arrows point to the same cells in corresponding pictures (not all cells are labeled in these transient transfections). Expression of TAM-67 did not influence the expression or distribution of β-catenin or the endogenous cadherin, compared to RA treatment. E-L. SK-BR-3 cells were transfected with c-jun and GFP. The jun plasmid is not epitope tagged and to detect jun-transfected cells we co-transfected GFP. GFP was detected by immunofluorescence (E, G, I, K), cadherin was detected by a pan-cadherin antibody (F, H), and β-catenin was detected by an anti β-catenin monoclonal antibody (J, L). Arrows point to the same cells in corresponding pictures. In control cells, GFP expression had no effect on cadherin or β-catenin staining or cell morphology in retinoid treated cells (E, F, I, J). Exogenous expression of c-jun did not influence cell morphology, cadherin or β-catenin expression (G, H, K, L).

Figure 4: Overexpression of β-catenin is not sufficient to induce a morphologic change. SK-BR-3 cells were transfected with vector alone (A, B), wild type β-catenin (C, D), or a mutant form of β-catenin, S37A that is more resistant to degradation (E, F) {1530}. In B, the cells were also treated with RA for 48 hours prior to fixation. The cells were fixed and stained for β-catenin (A-C, E) or cadherin (D, F). Arrows point to the same cells in corresponding pictures (C, D, E, F). RA dramatically increased the expression of β-catenin at sites of cell-cell contact in vector treated cells (Compare A to B). However, despite the high level of β-catenin expression there was no corresponding increase in cadherin expression, nor any change in cell morphology. Note that the cadherin images are overexposed to visualize the cells. SK-BR-3 cells were transfected with vector (pCDNA3) or with wild

type β-catenin (G). Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Overexpression of β-catenin is not sufficient to increase the expression of the endogenous cadherin, in contrast to retinoid treated cells. This was in contrast to β-catenin expression, which was significantly higher in the transfected cells, considering only about 10-20% of the cells were transfected.

Figure 5: E-cadherin expression is sufficient to mimic the effects of RA on cell morphology. SK-BR-3 cells were transfected with vector (A, B) or with a human E-cadherin construct, hecD pCDNA3 (C) or with wild type β-catenin (D). Cells were also cotransfected with a green fluorescent protein expression vector, and sorted by FACS to isolate the transfected cells. Cells were replated onto coverslips, grown +/- RA for 48 hours, then fixed. Cell morphology was determined by phase imaging. A, B) As expected, RA had a profound effect on cell morphology. E-cadherin expression was sufficient to mimic the RA-induced changes in cell morphology (C), but β-catenin expression was not (D). E-cadherin expression is also sufficient to mimic RA-induced increase in β-catenin. SK-BR-3 cells were transfected with a human E-cadherin construct, hecD pCDNA3 (E,F) or with the empty vector, pCDNA3 (G,H). The cells were then stained for E-cadherin (E,G) or β-catenin (F,H). Arrows point to the same cells in corresponding pictures. E-cadherin expression resulted in an increase in the expression of β-catenin. Both E-cadherin and β-catenin were localized to sites of cell-cell contact. ~~By~~ In contrast no E-cadherin ~~could be seen in~~ was present cells transfected with the empty vector, and there was no change in β-catenin expression or localization. I. SK-BR-3 cells were transfected with the empty vector, pCNDA3, with full length E-cadherin. An E-cadherin antibody raised against the E-cadherin extracellular domain revealed high expression of the full length E-cadherin. Expression of E-cadherin is sufficient to increase expression of β-catenin considering that only 10-20% of the cells were transfected.

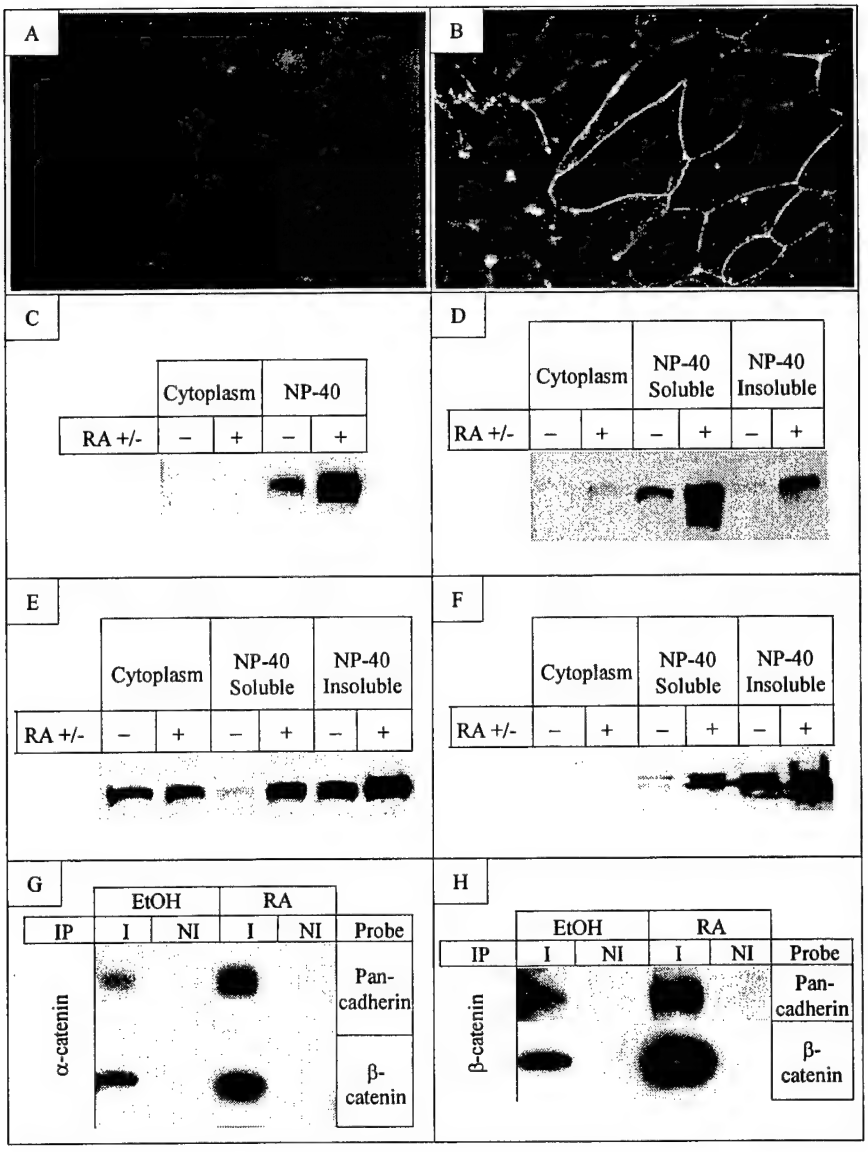
Figure 6. Calcium-dependent adhesion is not necessary to mediate the effects of RA on DNA-synthesis. A. SKBR3 cells transfected with the indicated plasmids and GFP were sorted by FACS and grown with or without RA for 48 h. Tritiated thymidine uptake was measured as described earlier ($$). B. SKBR3 cells were grown for 48 h in 2 mM or 50 uM Ca++ and tritiated thymidine uptake measured. Treatment of cells with hydroxyurea completely prevented tritiated thymidine uptake (not shown). Exogenous expression of E-cadherin did not significantly reduce DNA-synthesis and reduced calcium medium did not reverse the effects of RA.

Figure 7. Inhibition of calcium dependent cell-cell adhesion inhibits the ability of RA to decrease cytoplasmic β-catenin but does not affect β-catenin signaling. A. SKBR3 cells were transiently transfected with wild type or S37A mutant forms of β-catenin and cytoplasmic extracts prepared Untransfected SKBR3 cells expressed very low levels of β-catenin (not shown). Cytoplasmic β-catenin levels were markedly elevated following transfection. . RA-treatment reduced both wild-type and S37A cytoplasmic β-catenin levels. B. RA-treatment reduced cytoplasmic β-catenin levels in 2 mM Ca^{++} but not 50 uM Ca^{++}. C. SKBR3 cells were transfected with β-catenin and TOPflash. RA-treatment reduced reporter activity in normal and reduced calcium medium.

Figure 8. Schematic relating the three pathways affected by RA. In the present study we hypothesize that RA affects SKBR3 cell differentiation by increasing cadherin expression directly or indirectly through activation of the RARE pathway. This pathway is not required for inhibition of cell proliferation. Since SKBR3 cells have very low levels of β-catenin and are known to be growth inhibited by AP-1 blockade, we hypothesize that the growth inhibitory actions of RA in SKBR3 cells

are mediated via inhibition of AP-1. In other cells, which have low AP-1 and high levels of β-catenin signaling RA may regulate cell proliferation by inhibition of the β-catenin/TCF pathway. Although increased cadherin expression can decrease cytoplasmic β-catenin levels this mechanism is not absolutely required for RA to inhibit β-catenin/TCF signaling.

Pishvaian, Figure 1



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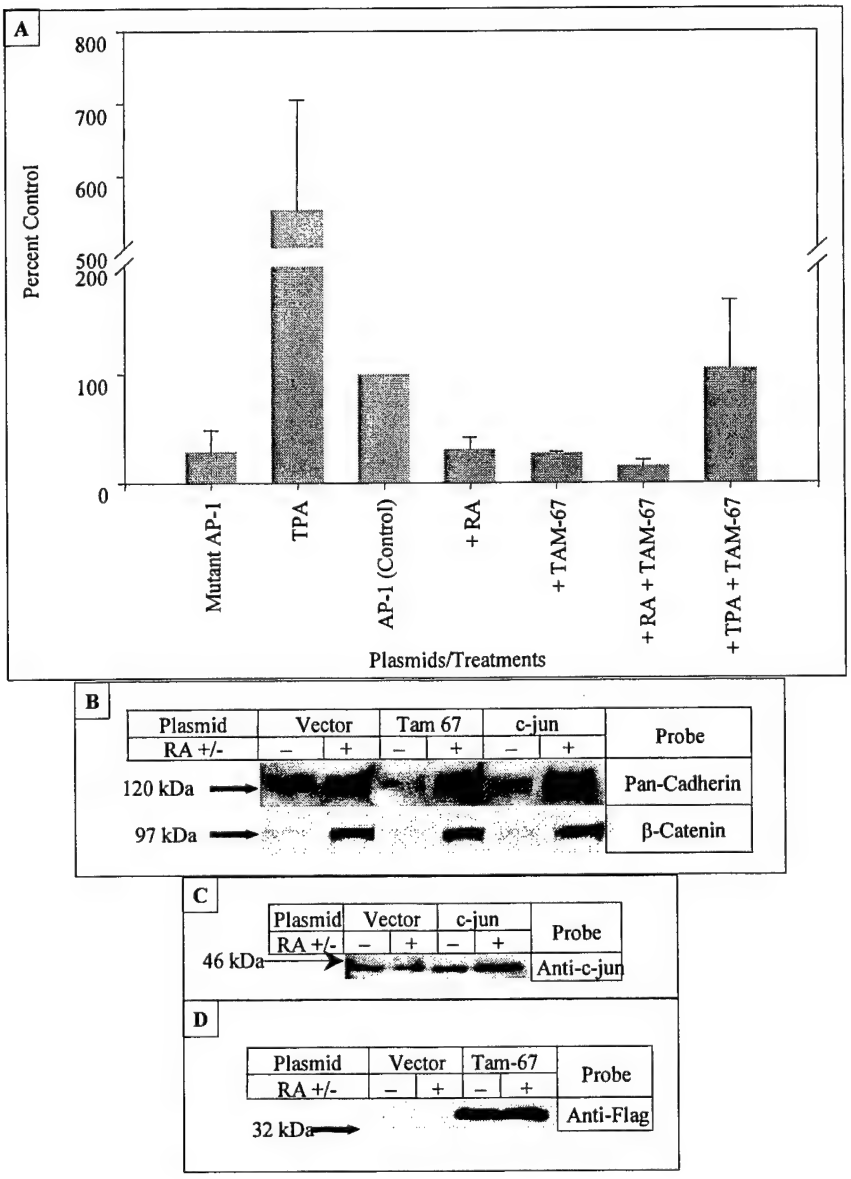

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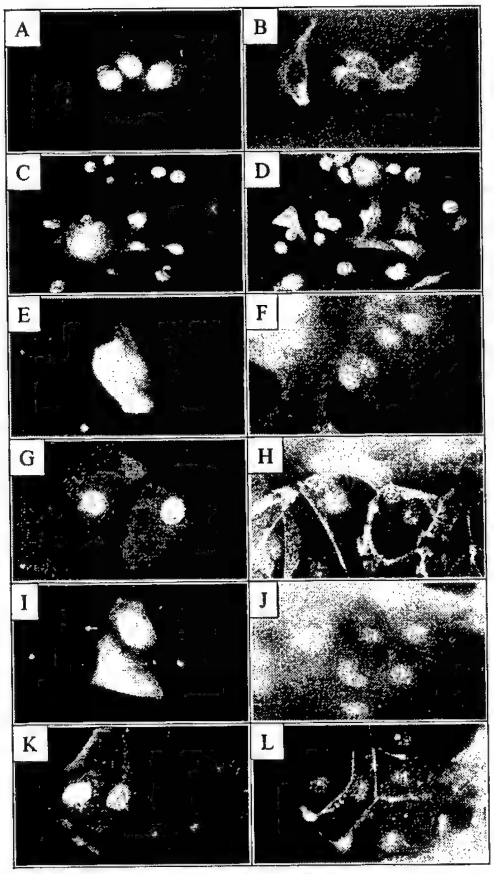
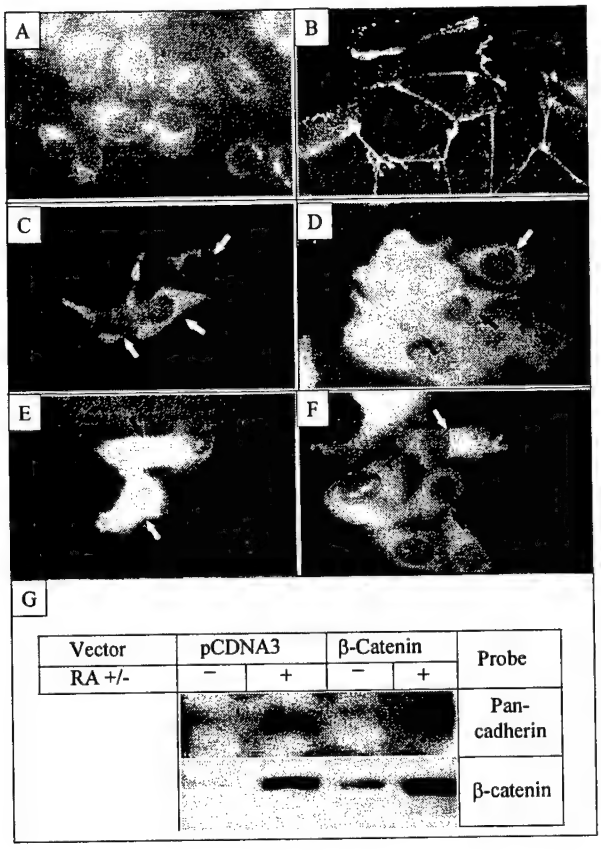


Pishvaian, Figure 4



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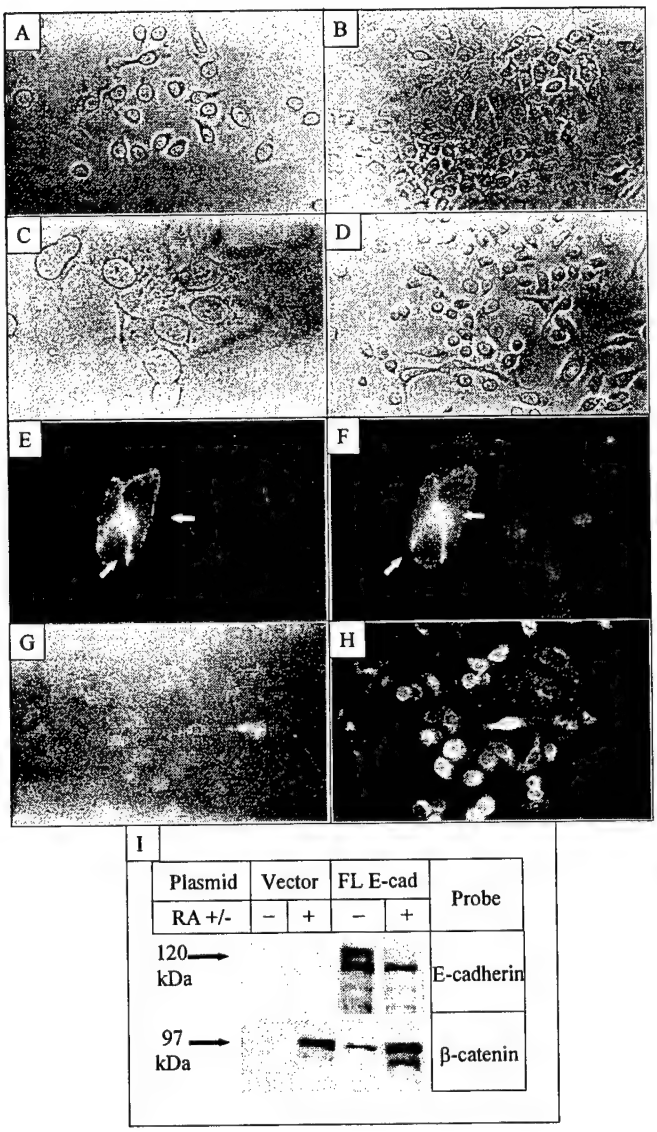

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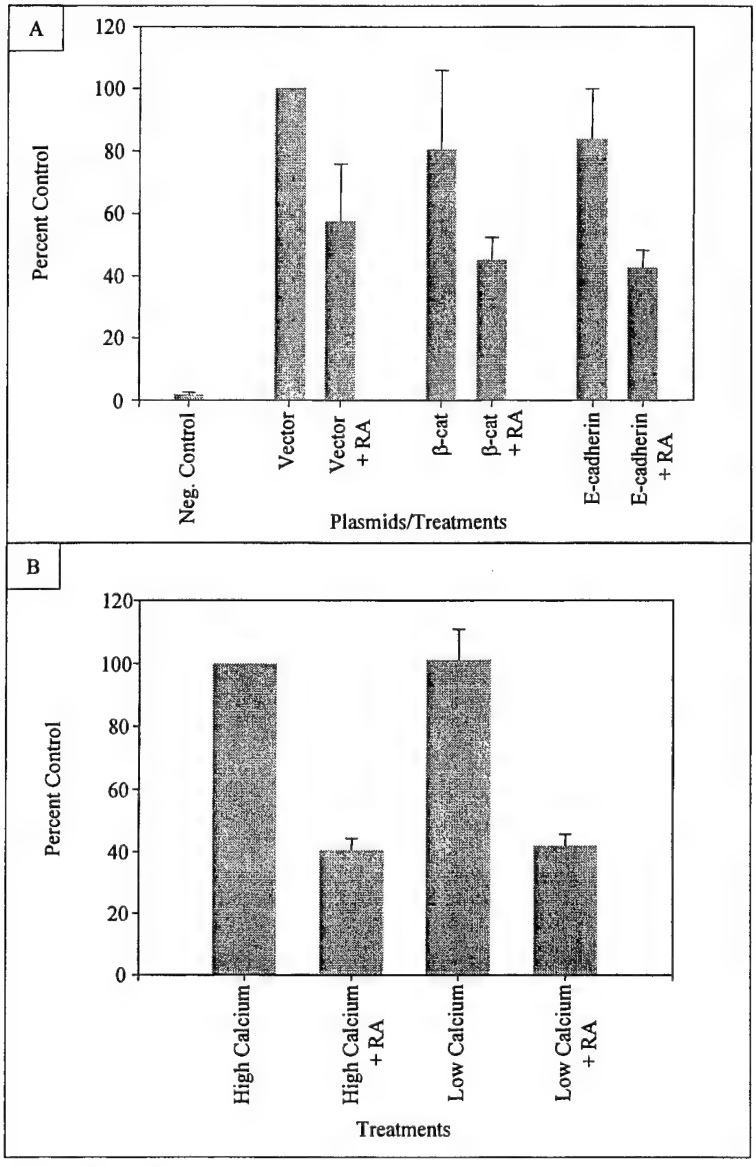

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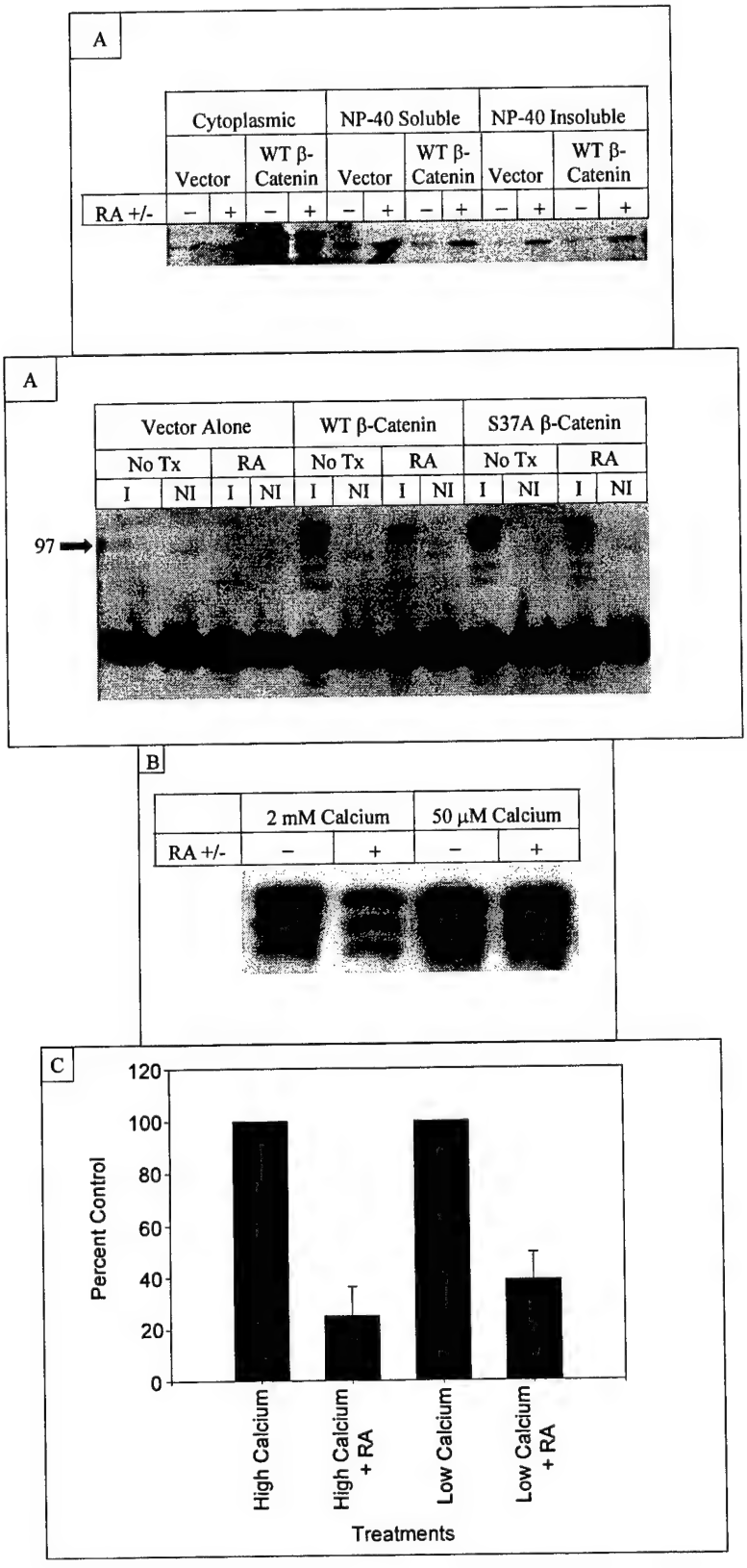

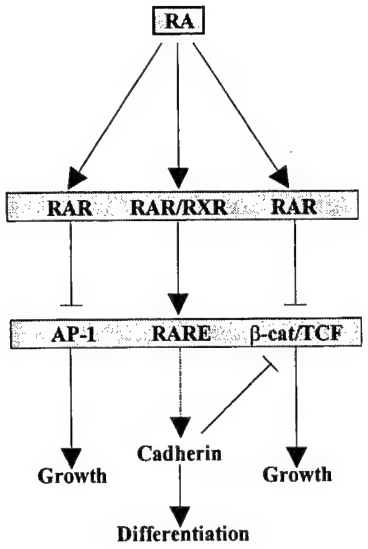
RA
RAR
RAR/RXR
RAR
AP-1
RARE
β-cat/TCF
Growth
Cadherin
Growth
Differentiation

FIGURE 8

Cross-regulation of β-catenin–LEF/TCF and retinoid signaling pathways

Vijayasurian Easwaran, Michael Pishvaian, Salimuddin and Stephen Byers

Vitamin A derivatives (retinoids) are potent regulators of embryogenesis, cell proliferation, epithelial cell differentiation and carcinogenesis [1]. In breast cancer cells, the effects of retinoids are associated with changes in the cadherin–β-catenin adhesion and signaling system [2,3]. β-catenin is a component of the Wnt signaling pathway, which regulates several developmental pathways [4]. Increases in cytoplasmic β-catenin and β-catenin signaling are also associated with numerous cancers, and are particularly important in colon cancer [5]. The oncogenic and developmental effects of β-catenin are mediated by its interaction with and activation of members of the LEF/TCF family of transcription factors [6–8]. Here, we show that retinoic acid (RA) decreases the activity of the β-catenin–LEF/TCF signaling pathway. This activity of RA was independent of the adenomatous polyposis coli (APC) tumor suppressor and ubiquitination-dependent degradation of cytoplasmic β-catenin. Consistent with this finding, β-catenin interacted directly with the RA receptor (RAR) in a retinoid-dependent manner, but not with the retinoid X receptor (RXR), and RAR competed with TCF for β-catenin binding. The activity of RA on RAR-responsive promoters was also potentiated by β-catenin. The data suggest that direct regulation of β-catenin–LEF/TCF signaling is one mechanism whereby RA influences development, cell differentiation and cancer.

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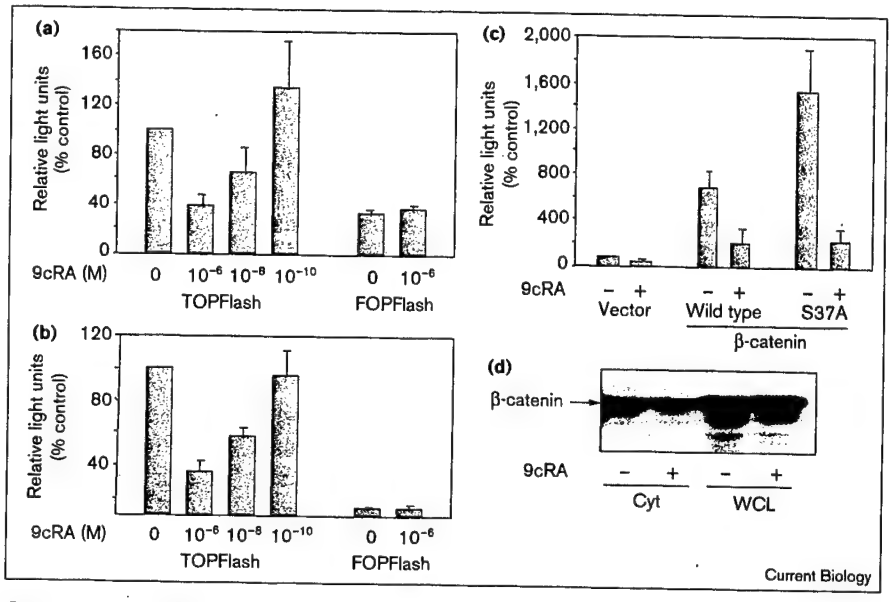
Results and discussion

Retinoid action is mediated through the retinoid receptor proteins RAR and RXR, which modulate gene expression directly, by binding RA-responsive elements (RAREs) and, indirectly, by inhibiting the activity of other transcription factor complexes such as AP-1 [1]. RXR also functions by heterodimerization with other members of the steroid hormone receptor superfamily, such as the thyroid hormone receptor, peroxisome proliferator activator receptor (PPAR) and the vitamin D receptor [9]. Many of the downstream pathways that mediate retinoid-induced changes in cells have yet to be defined. Increased cell–cell adhesion and the recruitment of cytoplasmic β-catenin to the membrane accompany the effects of 9-*cis*-RA (9cRA) on epithelial differentiation [2,3]. In contrast, elevated cytoplasmic β-catenin levels are associated with Wnt signaling, LEF/TCF-mediated transactivation and oncogenesis [6,10–13]. To investigate this potential connection, we first tested the ability of 9cRA to influence β-catenin signaling activity by measuring β-catenin–LEF/TCF-regulated luciferase reporter activity. We used 9cRA in these experiments because it can activate RXR homodimers in addition to RAR–RXR heterodimers. Figure 1a shows that, in MCF-7 breast cancer cells, which express wild-type adenomatous polyposis coli (APC), 9cRA reduced LEF/TCF reporter activity in a dose-dependent manner. Similar results were obtained with two other cell lines that are growth inhibited by RA (HS578t and SKBR3 cells; data not shown).

The β-catenin protein and signaling activity is controlled by APC-regulated serine phosphorylation and ubiquitin-dependent protein degradation [5,14]. We next tested whether the actions of RA on β-catenin–LEF/TCF signaling required APC, and β-catenin ubiquitination. Figure 1b shows that RA effectively inhibited LEF/TCF reporter activity in retinoid-sensitive APC-mutant colon cancer cells (Caco-2) and demonstrates that APC is not required for RA to inhibit β-catenin–LEF/TCF signaling. Similar results were obtained in another retinoid-sensitive APC-mutant cell line HT29 (data not shown). If the actions of RA involve the targeting of cytoplasmic β-catenin for ubiquitination and proteosomal degradation (in an APC-independent manner), one would anticipate that the signaling activity of stable, non-ubiquitinatable mutants of β-catenin would not be affected by RA. For example, the signaling activity of a β-catenin mutant in which the Ser37 residue is mutated to Ala (S37A) is resistant to inhibition by APC [14]. In contrast, Figure 1c shows that RA effectively inhibited LEF/TCF reporter activity induced by the S37A stable mutant form of β-catenin and further indicates that the effects of RA are unrelated to events that regulate β-catenin ubiquitination and proteosomal degradation [15]. Consistent with this, the cytoplasmic pool of β-catenin was unaffected by RA in Caco-2 cells (Figure 1d). These results show that, unlike APC and cadherins, the influence of RA on β-catenin signaling does not require a change in the signaling pool of β-catenin itself.

Figure 1



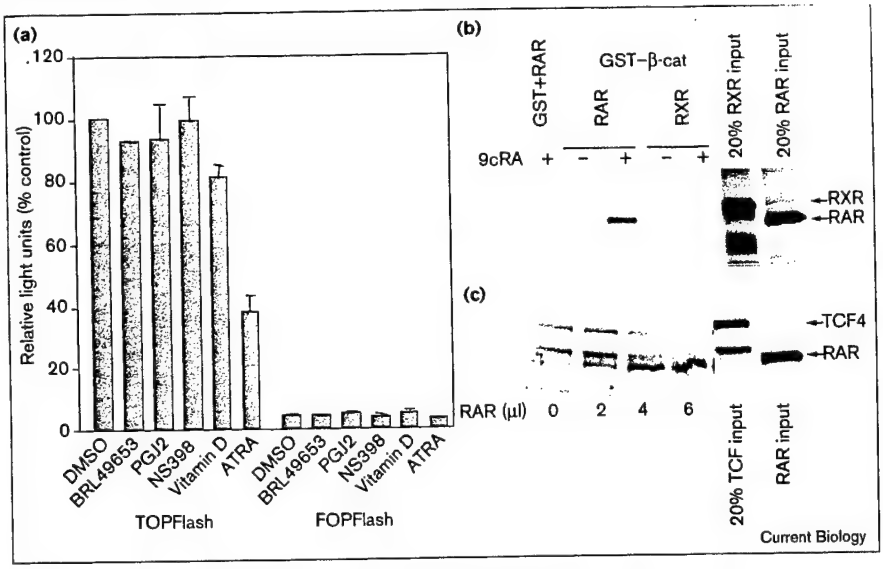
(a) Reduction of β-catenin–LEF/TCF signaling by 9cRA. MCF-7 cells transfected with the LEF/TCF reporters TOPFlash (optimal LEF/TCF-binding site) and FOPFlash (mutated LEF/TCF-binding sites) were treated with various doses of 9cRA or ethanol for 48 h. The experiment was repeated at least three times, with each treatment repeated in triplicate. Error bars represent standard deviation. Morphological analysis did not indicate significant cell death or toxicity. Similar results were obtained with HS578t and SKBR3 cells. **(b)** In APC-mutant Caco-2 cells, 9cRA decreases β-catenin–LEF/TCF signaling. Caco-2 cells were transfected with TOPFlash and FOPFlash and treated with 9cRA for 48 h. LEF/TCF reporter activity was monitored as described in the Supplementary material. Similar results were obtained with APC-mutant HT29 cells. **(c)** Signaling induced by wild-type β-catenin and the S37A non-ubiquitinatable form of β-catenin is reduced by 9cRA. MCF-7 cells were transfected with vector or wild-type β-catenin or the S37A mutant of β-catenin, and the LEF/TCF reporters. Cells were treated with 10^{-6} M 9cRA for 48 h. LEF/TCF reporter activity was monitored as described in the Supplementary material. **(d)** In Caco-2 cells, β-catenin protein levels are not altered by 9cRA. Caco-2 cells were treated with ethanol or 10^{-6} M 9cRA for 48 h and either lysed in sample buffer to make a whole cell lysate (WCL) or a cytoplasmic fraction (cyt) prepared as described in the Supplementary material.

To investigate the receptor specificity of vitamin A action on β-catenin–LEF/TCF signaling, we tested the role of PPAR and vitamin D receptors, two other steroid receptor families that are known to influence colon cancer [16]. Caco-2 cells were used in these experiments because they express PPAR, vitamin D and retinoid receptors, and respond to all of the cognate ligands [16–18]. Figure 2a shows that treatment of Caco-2 cells with three different PPAR ligands did not modulate β-catenin signaling activity. Vitamin D3 treatment exerted a small but consistent inhibitory effect on β-catenin signaling activity. In contrast, 9cRA (Figure 1) and all-*trans*-RA markedly decreased LEF reporter activity. Taken together, these data point to a direct effect of RA on the regulation of β-catenin–LEF/TCF-mediated transactivation, potentially mediated by RAR and/or RXR.

To test whether β-catenin could interact directly with retinoid receptors, glutathione-S-transferase (GST) 'pull-down' experiments were carried out. Figure 2b shows that, compared with GST alone, *in vitro* transcribed and translated RAR and RXR interacted slightly with a GST–β-catenin fusion protein. Importantly, the interaction of β-catenin with RAR, but not with RXR, was markedly increased by RA. The presence of RXR in the reaction did not inhibit or stimulate β-catenin–RAR interactions (data not shown). These data suggest that, for RA to regulate β-catenin–LEF/TCF reporter activity, it might modulate assembly of the β-catenin–LEF/TCF complex. To test this, we investigated the effects of RA and RAR on TCF4–β-catenin interactions. Figure 2c shows that, in the presence of RA, RAR did indeed reduce GST–β-catenin–TCF interactions. Significant inhibition of TCF4 binding was evident even in the presence of approximately equimolar amounts of RAR. These results indicate that RA-activated RAR competes with TCF for binding to β-catenin in a reaction that does not require RAR heterodimerization with RXR.

A direct interaction between β-catenin and RAR together with the ability of β-catenin to act as a co-activator for LEF/TCF suggests that β-catenin might regulate the activity of RAR-responsive promoters. We next tested the ability of β-catenin to augment the RA stimulation of RA-responsive promoters. Figure 3a shows that transient transfection of the S37A stable form of β-catenin into MCF-7 cells increased the activity of the RA-responsive RARβ promoter. Similar results were obtained using another RARE reporter but not with an estrogen-responsive reporter construct (Figure 3b,c). Like some other co-activators, β-catenin slightly activated RARE reporters in the absence of RA but this was always 10% or less of RA-stimulated values (data not shown). IκBα, the inhibitor of the transcription factor NFκB, can also interact with retinoid receptors, in this case with RXR, not RAR [19]. In contrast to β-catenin, however, IκBα inhibits RXRE reporter activity. Perhaps the documented ability of the β-catenin carboxyl terminus to recruit the transcriptional machinery allows RAR–β-catenin heterodimers to transactivate [20].

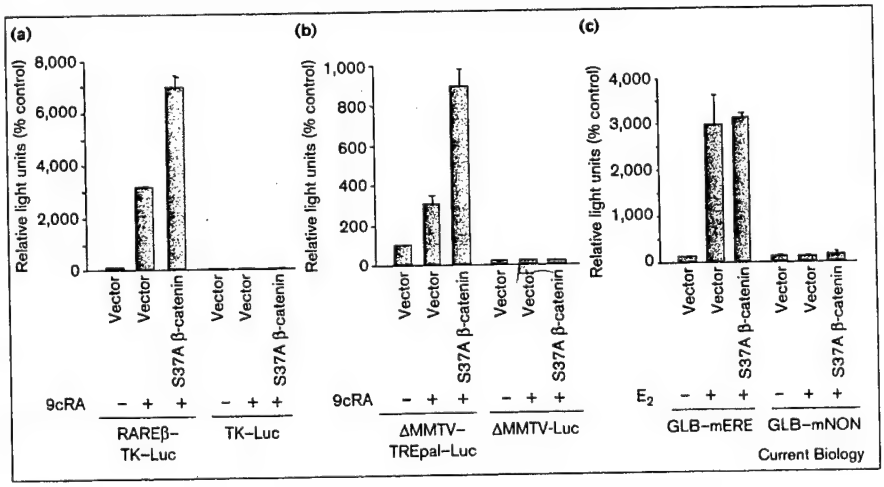
Figure 2



(a) Effect of various steroid-receptor-specific ligands on β-catenin–LEF signaling. Caco-2 cells were transiently transfected with the LEF/TCF reporters and treated with the indicated ligands (10^{-6} M) for 48 h. Three different PPAR ligands, BRL49653, PGJ2 and NS398, did not affect LEF/TCF reporter activity. Vitamin D3 exerted a small but consistent inhibitory affect. All-*trans*-RA (ATRA) markedly inhibited reporter activity. Reporter activity was measured as described in the Supplementary material. DMSO, dimethyl sulphoxide. **(b)** Interaction of β-catenin with RAR and RXR. *In vitro* transcribed and translated ^{35}S-labeled RARα and RXRα were incubated, in the absence (–) or presence (+) of 9cRA (10^{-6} M), with glutathione–agarose prebound with GST or GST–β-catenin for 30 min at 25°C. After washing, the protein interacting with β-catenin was eluted by adding 15 mM reduced glutathione, resolved on 12% gels by SDS–PAGE and detected using autoradiography. **(c)** RARα competes with TCF4 for binding to GST–β-catenin. Two TCF4 products were consistently observed after the transcription and translation reaction. The β-catenin-binding site of TCF is at the amino terminus [20]. Because both forms of TCF4 readily bound β-catenin, it is probable that the smaller species represents either a proteolytic product that is missing part of the carboxyl terminus, or usage of an alternative termination codon. The binding of both forms to β-catenin was decreased in the presence of RAR and RA. Arrows indicate the position of the *in vitro* transcribed and translated RAR, RXR and TCF4.

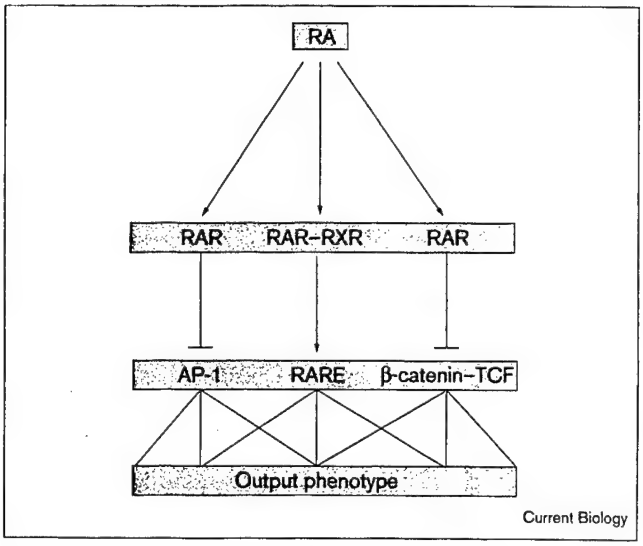
Retinoids are important signaling molecules, both in the adult and in the developing embryo, and RA can dramatically inhibit the development of anterior structures [21]. Wnt and its *Drosophila* homologue Wingless are also important in embryonic patterning, and overexpression of Wnt-1 or β-catenin in *Xenopus* results in anterior axis duplication [7,8,22]. Our demonstration that RA influences β-catenin–LEF/TCF signaling and that β-catenin influences RA signaling suggests that these two important developmental pathways might interact more directly than previously envisioned. Because increased levels of cytoplasmic β-catenin and/or increased β-catenin–LEF/TCF-signaling can transform cells, we propose that one mechanism whereby RA inhibits tumorigenesis might be by directly affecting β-catenin–LEF/TCF transactivation. If this is true, RA could be considered as a therapeutic agent for cancers in which β-catenin–LEF/TCF transactivation is overactive. In other studies, we showed that RA treatment of some breast cancer cells increases cadherin expression and function [2]. Increased cadherin expression

Figure 3



RARE-dependent transactivation is augmented by β-catenin. MCF-7 cells were transfected with the indicated luciferase (luc) reporter plasmids and a construct encoding the S37A mutant form of β-catenin. **(a,b)** In the presence of 10^{-6} M RA, a stable S37A form of β-catenin augmented the ligand-induced transactivation of two RARE reporters ((a) RAREβ and (b) ΔMMTV-TREpal). **(c)** In the presence of 10^{-9} M estradiol (E_2), β-catenin did not augment ERE-dependent reporter transactivation (GPB-mERE). See Supplementary material for a description of the various reporters.

Figure 4



Three pathways affected by RA. The ability of RA to influence AP-1 and β-catenin–LEF/TCF signaling depends on RAR but not RXR, whereas the ability of RA to influence RARE activation requires RAR–RXR heterodimer formation. The output phenotype then depends on the genetic program that is activated by the combined effects of AP-1 and/or β-catenin–LEF/TCF inhibition and RARE activation. The contribution from each pathway will vary depending on the levels of the reactants. In colon cancer cells, which have very high levels of β-catenin, RA-activated RAR might preferentially affect this pathway.

can also modulate β-catenin signaling, by depleting the cytoplasmic pool of β-catenin [23,24]. Depending on the cellular context, therefore, RA can increase the adhesive function of β-catenin and independently decrease its signaling activity. Taken together with the effects of RA on other known pathways, we suggest that the pleiotropic and context-dependent effects of RA result from the differential regulation of AP-1, RARE and β-catenin–LEF/TCF-activated transcriptional programs (Figure 4).

Supplementary material

Supplementary material including additional methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

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Exogenous Expression of β-Catenin Regulates Contact Inhibition, Anchorage-independent Growth, Anoikis, and Radiation-induced Cell Cycle Arrest

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Abstract. β-Catenin is an important regulator of cell–cell adhesion and embryonic development that associates with and regulates the function of the LEF/TCF family of transcription factors. Mutations of β-catenin and the tumor suppressor gene, adenomatous polyposis coli, occur in human cancers, but it is not known if, and by what mechanism, increased β-catenin causes cellular transformation. This study demonstrates that modest overexpression of β-catenin in a normal epithelial cell results in cellular transformation. These cells form colonies in soft agar, survive in suspension, and continue to proliferate at high cell density and following γ-irradiation. Endogenous cytoplasmic β-catenin levels and signaling activity were also found to oscillate during the cell cycle. Taken together, these data demonstrate that β-catenin functions as an oncogene by promoting the G_1 to S phase transition and protecting cells from suspension-induced apoptosis (anoikis).

Key words: β-catenin • oncogene • cell cycle • anoikis • apoptosis

β-Catenin is a 92–97-kD protein associated with the intracellular tail of the intercellular adhesion molecule E-cadherin (Ozawa et al., 1989). Through this association, β-catenin plays an important role in strong cell–cell adhesion as it links E-cadherin (and other members of the cadherin family) to the actin cytoskeleton through the protein α-catenin (Hirano et al., 1992; Kemler, 1993). One mechanism by which cell–cell adhesion can be negatively regulated is via the phosphorylation of β-catenin on tyrosine residues (Behrens et al., 1993). There are some indications that this may be an important event in the transition from a benign tumor to an invasive, metastatic cancer (Sommers et al., 1994).

β-Catenin is also a regulator of embryogenesis, a role that was first suspected when it was shown to be the mammalian homolog of the *Drosophila* segment polarity gene *Armadillo* (Peifer et al., 1992). Further studies in *Drosophila* and *Xenopus* have revealed that β-catenin is a component of the highly conserved Wnt/Wingless signal transduction pathway that regulates body patterning in both species (Peifer, 1995; Gumbiner, 1997).

The membrane-associated and cytoplasmic pools of β-catenin have disparate activities: adhesion and signaling, respectively. The accumulation of cytoplasmic β-catenin drives its interaction with members of the LEF/TCF family of nuclear transcription factors that results in altered gene expression, which is the transduction of the Wnt/Wg signal (Clevers and van de Wetering, 1997). This accumulation of cytoplasmic β-catenin is regulated at the level of its degradation (Peifer et al., 1994; Peifer, 1995; Papkoff et al., 1996). In the absence of the Wnt/Wg signal, phosphorylation of specific serine residues on β-catenin leads to its ubiquitination and degradation, removing it from the cytoplasm (Orford et al., 1997). Mutations of these serine residues inhibit the ubiquitination of β-catenin, which causes it to accumulate and signal constitutively (Morin et al., 1997; Orford et al., 1997).

Along with its position in a growth factor signaling pathway, the demonstration of an interaction between β-catenin and the product of the tumor suppressor gene, adenomatous polyposis coli (APC)[1], suggests that it is involved in oncogenesis (Rubinfeld et al., 1993; Peifer, 1997). Tumor cell lines with a loss of one copy of APC, and harboring mutations in the other allele, have high lev-

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1. *Abbreviations used in this paper:* APC, adenomatous polyposis coli; CON, control; EMT, epithelial to mesenchymal transition; FAK, focal adhesion kinase; HA, hemagglutinin epitope; ILK, integrin-linked kinase; PKC, protein kinase C; S37A, S37A mutant β-catenin plasmid; WT, wild-type β-catenin plasmid.

els of cytoplasmic (signaling) β-catenin, which is markedly reduced when functional APC is reintroduced (Munemitsu et al., 1995). Importantly, all mutant forms of APC found in human cancers are unable to reduce β-catenin levels in these cells. The importance of elevated β-catenin in human cancer was further substantiated when mutations in the β-catenin gene were described in colon cancer and melanoma cell lines (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). At least one of these mutations results in a more stable form of the protein.

A retroviral insertion screen for oncogenes using the NIH-3T3 cell line also implicated β-catenin as a possible oncogene, as the insertion of the retrovirus resulted in the expression of a β-catenin protein that lacked the NH_2 terminus (Whitehead et al., 1995). In contrast, overexpression of a stabilized form of β-catenin is unable to mimic the morphological effects of Wnt-1 in fibroblasts (Young et al., 1998).

Although much is now known about this signaling system, the actual cellular processes in which β-catenin plays a regulatory role is unclear. As described above, it regulates cadherin-mediated cell–cell adhesion. Although it appears to regulate gene expression, few target genes have been demonstrated. Based on its relationship with Wnt and APC, it is possible that β-catenin may positively regulate cellular proliferation or inhibit apoptosis. It is also tempting to speculate that the adhesive and the putative oncogenic functions of β-catenin are related and that it may be, at least in part, the mechanistic link between cell–cell adhesion, contact inhibition, and/or apoptosis. However, no studies have directly tested the hypothesis that β-catenin is actually oncogenic.

This report utilizes the MDCK cell line to determine the impact of overexpressing wild-type or a stabilized mutant form of β-catenin in nontransformed epithelial cells. The data demonstrate that β-catenin alters cell cycle progression and confers enhanced growth in soft agar, a surrogate marker for tumorigenicity. In addition, β-catenin confers resistance to suspension-mediated apoptosis (anoikis), radiation-induced cell cycle arrest, and allows cells to continue cycling when cultured at confluence. In short, β-catenin functions as an oncogene in the MDCK normal epithelial cell line.

Materials and Methods

Cells, Plasmids, and Stable Transfections

MDCK cells are a canine kidney-derived nontransformed epithelial cell line that are maintained in DME (GIBCO BRL), supplemented with 5% FBS. A1N4 cells are a human mammary nontransformed epithelial cell line that are grown in IMEM, supplemented with 0.5% FBS, 0.5% hydrocortisone, 5 μg/ml insulin, and 10 ng/ml EGF (Stampfer and Bartley, 1988). These cells synchronize in G_0 in the absence of EGF. The wild-type (WT) and S37A mutant (S37A) β-catenin plasmids were described previously (Orford et al., 1997). The bacterial chloramphenicol acetyltransferase gene driven by the CMV promoter of the pcDNA 3 plasmid (Invitrogen Corp.) served as the negative control (CON). For stable transfections, 800,000 MDCK cells were plated per 100-mm tissue culture plate. The next day, 15 μg of the various plasmids were transfected using the lipofectamine PLUS method (GIBCO BRL): 32 μl lipofectamine and 45 μl PLUS reagent. All of the plasmids included the neomycin-resistance cassette for selection. 48 h later, the cells were split 1:20 and cultured for 2 wk in the presence of 500 μg/ml of Geneticin (GIBCO BRL). An approximately equal number of colonies grew up for each transfected plasmid. For each transfection, all of the colonies were trypsinized and combined to give stable cell pools.

Immunoblotting

Whole cell and cytoplasmic lysates were made and immunoblotting performed as described previously (Orford et al., 1997).

Immunofluorescence

Cells were grown to confluence in 4-well BIOCOAT chamber slides (Falcon Plastics). Cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Cells were then permeabilized in 0.2% Triton X-100, 4% paraformaldehyde in PBS for 10 min. After washing in PBS, cells were blocked in 3% ovalbumin for 1 h. The chambers were incubated with primary antibodies overnight at 4°C. After washing in PBS five times for 5 min each, fluorescein- or Texas red-conjugated secondary antibodies were added for 1 h. Primary and secondary antibodies were diluted in 6% normal goat serum. After removal of the secondary antibody, the chambers were washed five times for 5 min in PBS, and the chambers removed. The cells were mounted with Vectashield (Vector Labs, Inc.).

Antibodies

The anti–β-catenin (C19220) and anti-p27 (K25020) mAbs were from Transduction Laboratories. The antihemagglutinin mAb (HA-11) was purchased from Berkeley Antibody Co., Inc. A second high affinity anti-HA mAb was purchased from Boehringer Mannheim Corp. (#186723). The anti–E-cadherin (SHE78-7) mAb was purchased from Zymed Labs, Inc. Peroxidase- and fluorescein-labeled secondary antibodies were purchased from Kirkegaard and Perry Laboratories, Inc. The Texas red-labeled secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc.

β-Catenin–LEF/TCF Signaling Assays

In 12-well dishes, cells were transfected with 0.5 μg of the TOPFLASH LEF/TCF reporter plasmid (van de Wetering et al., 1997) and 0.005 μg of the constitutively expressed Renilla luciferase, as a normalization control. As a negative control, cells were transfected with the FOPFLASH reporter plasmid in which the LEF/TCF binding sites have been mutated. The cells were lysed and assayed for Firefly and Renilla luciferase activities using the STOP & GLO assay (Promega Corp.). All results are normalized to the Renilla luciferase activity.

Soft Agar Growth Assay

For each cell pool, 150,000 cells were suspended in 3 ml DME + 5% FBS, and warmed to 37°C. 300 μl of a prewarmed (52°C) 3% agarose/PBS solution was mixed with the cell suspension and then layered into 3 wells of a 6-well plate (1 ml/well), which were previously coated with 1 ml of 0.6% agarose in DME. The agar was allowed to solidify at room temperature for 20 min before 3 ml of growth medium was added to each well. The medium was changed every three days. After 14 d, the colonies were counted by an Omnicon 3600 Colony Counter and photographed.

Growth Curves

To have an equal number of cells plated at the first time point, 10,000 CON, and 5,000 WT and S37A cells were plated per well of 12-well plates. At each time point, the cells were washed once in PBS and trypsinized in 1 ml trypsin/versene (GIBCO BRL). The single cell suspension was counted on a Coulter Counter set at 10 μm min with 20-μm maximum diameter. Each data point was performed in triplicate.

Plating Efficiency Assay

For each cell pool, 100 cells were plated onto each of three 100-mm tissue culture dishes in DME + 5% FBS. 4 d after plating, the colonies were photographed at 400×. After 8 d, the cells were washed with PBS, stained with crystal violet, and washed with water. The colonies were counted and then photographed. The plating efficiency is the mean number of colonies per dish/100 cells plated per dish.

Quantification of Cell Shedding

Cells were cultured in 6-well plates 3 d after confluence. The cells were

washed twice in PBS and 2 ml of fresh medium was added to each well. 24 h later, the shed cells were removed with medium and counted on a Coulter Counter, as described.

Cell Cycle Analyses

Two flow cytometric assays were used.

Vindelov Method. Cells were washed in PBS and trypsinized. Cells were washed in PBS and pelleted. After removing the wash buffer, the pellet was vortexed and resuspended in 0.1 ml of citrate/DMSO buffer (250 mM sucrose, 40 mM $Na_3C_6H_5O_7$ $2H_2O$, 5% DMSO, pH 7.60). The pellets were then frozen at −80°C. The cells were then processed as in Vindelov et al. (1983).

Ethanol Fixation Method. Cells were washed once in PBS and trypsinized. Trypsinized cells were pelleted at 1000 *g* and washed in 5 ml cold PBS. After a second centrifugation, the cells were resuspended in 0.5 ml cold PBS and fixed by dripping in 1.5 ml cold 100% ethanol, while slowly vortexing the cell suspension. After at least 1 h at 4°C, the cells were stained with propidium iodide and DNA content was measured by flow cytometry. The ethanol fixation method was also used for the flow cytometric analysis of apoptosis.

Cell Synchronization Experiments

β-Catenin Protein Level. A1N4 cells were plated in 100-mm tissue culture dishes and grown overnight to ~40% confluency. The cells were washed three times in PBS and then maintained in the absence of EGF for 46–50 h. This synchronized >95% of the cells in the G_0/G_1 phase of the cell cycle. To stimulate reentry into the cell cycle, EGF-containing medium was added back to the cells. Parallel dishes were analyzed at each time point for β-catenin protein (whole cell or the cytoplasmic pool) and for the cell cycle distribution.

β-Catenin–LEF/TCF Signaling. 50,000 A1N4 cells were plated per well of 12-well dishes and transfected with 1 μg of the TOPFLASH reporter plasmid and 0.01 μg of the Renilla control plasmid by the calcium phosphate method. The cells were then synchronized by EGF starvation (G_0/G_1) or 1 μM nocodazole (G_2/M), or treated with the proteosomal inhibitor ALLN, which stabilizes β-catenin. The cells were collected and the luciferase measurements were made as described.

Anoikis Assays

Confluent cells were trypsinized into a single cell suspension. 700,000 cells were plated in 150-mm tissue culture dishes coated with 0.8% agarose, to which they could not attach. At the various time points, the cells were collected, washed in PBS, and any cell aggregates were dispersed by trypsinization. Cells were then analyzed for apoptosis using three separate assays.

DNA/Flow Cytometry. Samples were analyzed by flow cytometry (see Cell Cycle Analyses, Ethanol Fixation). In this analysis, the hypodiploid peak constituted the apoptotic population.

AnnexinV Labeling. Samples were stained with fluorescein-labeled AnnexinV and propidium iodide (Trevigen) according to the manufacturer's protocol, and analyzed by flow cytometry. The two AnnexinV positive quadrants of the analysis were taken as the apoptotic fraction.

Hoechst Staining. Cells were fixed in 10% formalin for 10 min and stained with Hoechst #33258 (25 μg/ml in PBS) for 10 min at room temperature in the dark. Cells were placed on a glass slide and analyzed by fluorescence microscopy.

γ-Irradiation

750,000 CON, and 500,000 WT and S37A cells were plated in T75 tissue culture dishes. 26 h later, the flasks were exposed to 5 Gy of γ-irradiation. Another group of flasks received a mock irradiation (0 Gy). At 8 and 24 h after irradiation, the cells were trypsinized and their cell cycle profile was determined.

Results

Expression of β-Catenin Transgenes in MDCK Cells

To investigate the effects of β-catenin on normal cellular function, MDCK cells were stably transfected with constitutively expressed β-catenin transgenes that have been engineered to contain a COOH-terminal HA tag. In addition to WT β-catenin, a construct harboring a previously described serine to alanine point mutation at residue 37 (S37A) was used, which encodes for a β-catenin protein largely resistant to ubiquitination (Orford et al., 1997). The cells used are pooled stable transfectants; that is, after selection with G418, all of the drug resistant colonies resulting from each transfection were combined. These will be referred to as cell pools. As a negative control, a cell pool expressing the bacterial chloramphenicol acetyl transferase gene was generated (CON). Stable cell pools were generated to avoid the phenotypic artifacts that can result from the selection and propagation of individual clones derived from single transfected cells. We found that MDCK cells are especially prone to clonal morphological variation.

When examined by immunoblotting, expression of the HA tag was detectable only in the cell pool expressing the more stable S37A mutant (Fig. 1, B–E). We believe that epitope inaccessibility and antibody insensitivity result in the poor detection of the HA-tagged β-catenin and, consequently, the HA tag was undetectable by immunoblotting in untreated WT cells. To demonstrate that the WT cells were capable of expressing HA-tagged β-catenin, all three cell pools were treated with the histone deacetylatase inhibitor sodium butyrate to nonspecifically increase gene expression. This treatment resulted in clearly detectable expression in the WT cells and very high expression in the S37A cells, whereas the CON cells lacked expression under both conditions. Sodium butyrate treatment was not used in any other experiments in this study. In untreated cells, a similar pattern was seen by immunofluorescence microscopy. Using an antibody specific for the HA tag and a fluorescein-labeled secondary antibody, staining was detectable in the S37A cell pool (Fig. 1 E), but was difficult to detect in the WT cells (data not shown). To demonstrate the HA tag in the WT cells, a high affinity anti-HA antibody (Boehringer Mannheim) and a Texas red-conjugated secondary antibody was used to increase the sensitivity of the assay. Under these conditions, expression of the HA-tagged protein was clearly demonstrable in most of the WT cells (Fig. 1 D), even in the absence of butyrate, whereas expression was not evident in the CON cells (Fig. 1 C). A β-catenin specific antibody revealed a normal staining pattern in all three cell pools (Fig. 1, F–H).

Whole cell lysates do not exhibit any significant increase in total β-catenin levels (data not shown) because MDCK cells express a large amount of endogenous β-catenin, most of which is complexed with E-cadherin at the cell membrane. However, it is the cytoplasmic pool that is involved in β-catenin signaling and an increase in this pool was evident in both WT and S37A expressing cells, as compared with the CON cell pool (Fig. 1 A).

To confirm that β-catenin was being functionally overexpressed in both the WT and S37A cell pools, LEF/TCF-dependent nuclear signaling was measured using the TOPFLASH reporter construct (van de Wetering et al., 1997). This reporter consists of four consensus LEF/TCF binding sites placed upstream of the cFos minimal promoter. As a negative control, a similar reporter construct (FOPFLASH), in which the LEF/TCF binding sites have been

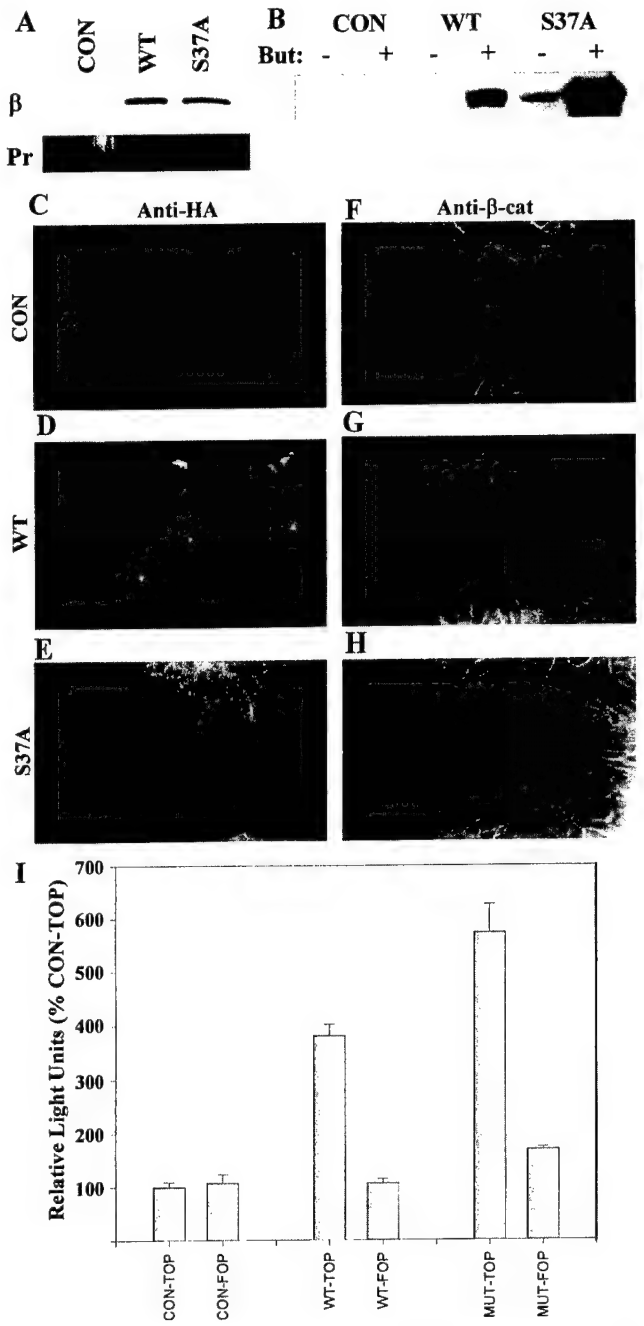

Figure 1. Expression of transgenes in MDCK stable cell pools. A, Equal protein from cytoplasmic extracts of CON, WT, and S37A cell pools was immunoblotted with an anti–β-catenin antibody. Amino black staining of nitrocellulose membrane demonstrates equal protein loading. B, Expression of HA-tagged β-catenin was determined by immunoblotting equal protein from whole cell lysates of the three cell pools cultured with and without sodium butyrate (But; to enhance gene expression) with anti-HA antibody (HA-11; BabCo). C–E, HA-tagged β-catenin can be detected in the WT (D) and S37A (E) cell pools by immunofluorescence. The HA-tag is absent in the CON cell pool (C). F–H, Expression of β-catenin in the same cell pools. I, β-catenin signaling activity was determined with the TOPFLASH LEF/TCF-responsive reporter construct. β-Catenin–LEF/TCF signaling is elevated above CON in both the WT and S37A cells. The negative control FOPFLASH reporter is essentially unaffected by β-catenin transfection.

mutated, was used. Even though the HA tag was not easily detected in the untreated WT cell pool, LEF/TCF signaling is elevated well above the control (Fig. 1 I, CON) in both the WT and S37A cell pools.

β-Catenin Overexpression Alters Cell Morphology

Overexpression of β-catenin in MDCK cells previously was shown to alter cell morphology. The stable cell pools used in this report have essentially the same morphology as the MDCKs expressing an inducible form of NH_2 terminally truncated β-catenin (Barth et al., 1997). The WT and S37A cell pools are less efficient at forming tight colonies of cells, as compared with CON cells (Fig. 2). In addition, the cells along the edges of the WT and S37A colonies tend to extend projections more readily, giving them a more mesenchymal morphology. The morphology of these cell pools also varied at high density. In contrast to their appearance at lower density, the WT and S37A cells appeared to be more tightly adherent to each other (data not shown). This is supported by the fact that these cells are significantly slower to round up when trypsinized during normal cell passaging. To confirm that expression of the β-catenin transgenes did not prevent strong intercellular adhesion, the ability of the WT and S37A cells transepithelial resistance was measured in the presence and absence of Ca^{2+}. Both the WT and S37A cells formed a strong barrier in the presence of Ca^{2+} (>1,000 ohms/chamber) that was completely diminished in the absence of Ca^{2+}. These results are consistent with what is seen in normal epithelial cell lines and confirms strong cadherin-mediated adhesion.

β-Catenin Stimulates Cell Proliferation

To characterize the distribution of these cells in the cell cycle, DNA/flow cytometry analysis was performed on these cells during exponential growth phase. Both of the β-catenin overexpressing cell pools had a reduced proportion of G_0/G_1 cells and an increased proportion of S and G_2 cells, as compared with the control cells (Fig. 3 A). This suggests that either a greater proportion of the WT and S37A cells are cycling or the G_1 phase of the cycle is shorter in duration than it is in the CON cells.

Growth curves demonstrated a significant difference between the β-catenin overexpressing cells (WT and S37A) and the CON cells (Fig. 3 B). The curves depicting the growth of the WT and S37A cell lines diverged from that of the CON cells, demonstrating that the alterations in cell cycle distribution resulted in increased growth. Also, overexpression of β-catenin increased saturation density of these cells (Fig. 3 B, inset). Together with the demonstration that the WT and S37A cells proliferate more rapidly at confluence (Fig. 4), it is clear that β-catenin overexpression significantly diminishes the property of contact inhibition of growth.

Interestingly, in every replication of this experiment, the number of cells in the WT and S37A wells was elevated (up to 50%) above the CON cells at the first time point of the growth curve. To determine if a difference in plating efficiency might explain the discrepancy in the cell number on the first day of the growth curves, 100 cells were plated per 100-mm tissue culture dish in three dishes for each cell pool. The colony count provides a rough estimate of the

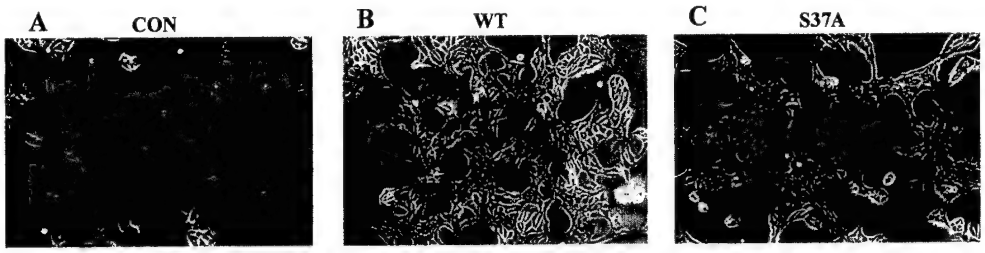

Figure 2. Exogenous β-catenin expression alters morphology of MDCK cells. Phase-contrast photographs of CON (A), WT (B), and S37A (C) cell pools demonstrate the effect of β-catenin overexpression on MDCK cell pools. β-Catenin-expressing cells show a more spindly, mesenchymal, less cell–cell adhesive morphology compared with the control cells.

plating efficiency of the cells. This experiment revealed a small (but not statistically significant) difference in plating efficiency that may contribute to the consistent differences in cell number, but does not explain them entirely (Fig. 3 C). We believe that the combination of increased plating efficiency and elevated proliferation rate account for the differences seen at the first time point.

An obvious increase in the rate of colony growth in the β-catenin overexpressing cells was more dramatic. The colonies from the WT and S37A cells were many fold larger than those from the CON cells. The morphology of these clones provides one explanation for the difference in colony size (Fig. 3, D, E, and F). Whereas the CON cells formed tightly adhesive, epithelioid colonies (Fig. 3 G), the WT and S37A cells formed a large number of colonies containing a more scattered, mesenchymal phenotype (Fig. 3 H). The morphological changes suggest that enhanced motility may contribute to this dramatic increase in colony size, but this is speculative. Also, the reduced adhesiveness in the WT and S37A cells may promote large colony formation by avoiding the contact inhibitory effect of tight cell–cell adhesion. In addition, other data suggest that the WT and S37A cells have an increased proliferative rate, even in the presence of strong intercellular adhesion (Fig. 4).

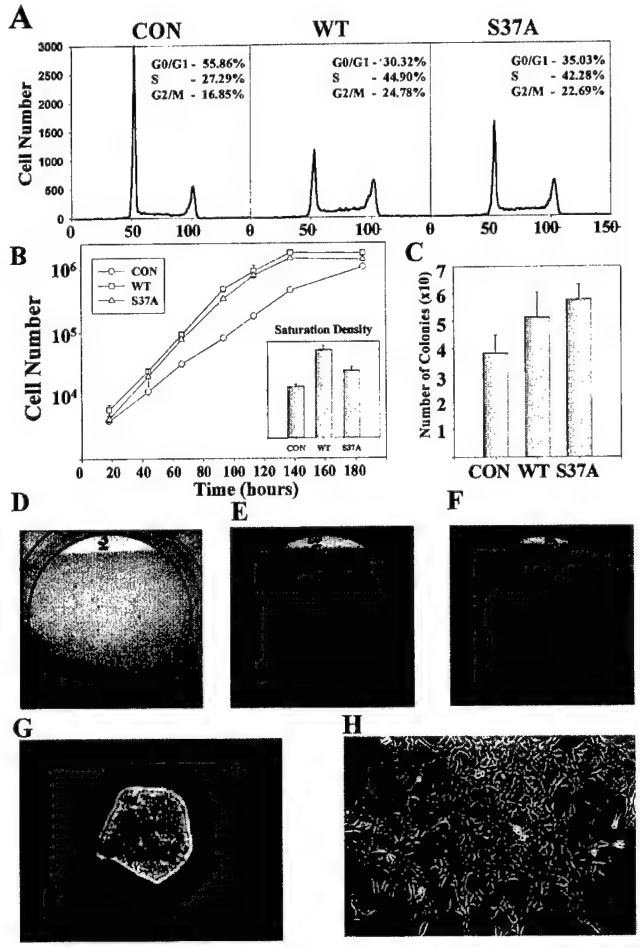

Figure 3. β-Catenin overexpression alters proliferation, plating efficiency, and colony morphology. A, DNA/flow cytometric analysis of the three cell pools during exponential growth demonstrates that the WT and S37A cell pools have a significantly lower percentage of cells in the G_0/G_1 phase of the cycle and a higher percentage in both S and G_2 phases of the cell cycle. B, Growth curves reveal that WT and S37A cells proliferate more rapidly than CON cells. To have approximately equal numbers of cells at time 0, 10,000 CON, 5,000 WT, and 5,000 S37A cells were plated per well in 12-well tissue culture plates. Each time point was done in triplicate. Graphing and SD calculations were performed with Sigmaplot. Error bars are hidden by symbols at several time points. Inset, saturation density of the three cell pools. Cells were counted at absolute confluence in 12-well plates. Each measurement is the mean of the cell counts from at least six wells. Graphing and SD calculation was performed with Sigmaplot. C–H, Plating efficiency assay reveals changes in colony morphology. 100 cells from each of the cell pools were plated in 100-mm dishes. After eight days, the colonies were stained with crystal violet, counted, and photographed. C, Number of colonies counted for each of the three cell pools. D–F, Photographs of crystal violet stained CON (D), WT (E), and S37A (F) colonies. G and H, Phase-contrast photographs of representative colonies from the CON (G) and WT (H) cell pools at four days. S37A colonies looked identical to the WT colony pictured. All experiments were performed at least three times with consistent and repeatable results.

β-Catenin Promotes Proliferation at High Cell Density

The reduction in proliferative rate that nontransformed cells experience at high cell density has been termed contact inhibition of growth. Although this is a widely recognized phenomenon, the signaling mechanisms involved remain unknown. To address this, the MDCK cell pools

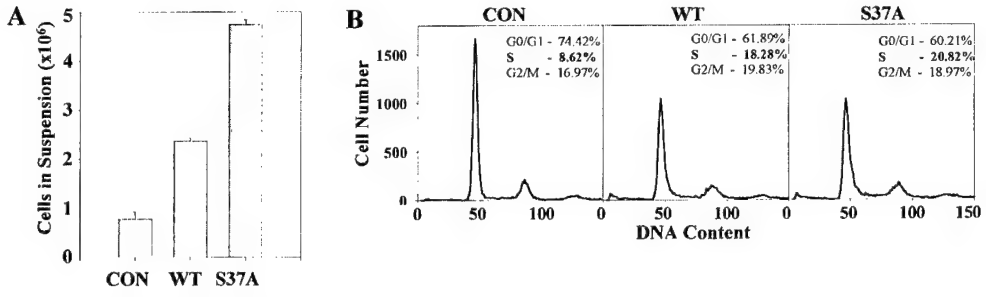

Figure 4. β-Catenin overexpression promotes proliferation at high cell density. A, Cells were cultured three days after confluence. After washing, cell shedding was measured over a 24 h period by counting the number of cells suspended in the medium. Graphing and SD calculation were performed using Sigmaplot. B, Cell cycle profiles of cells grown three days after confluence. After cells were washed twice with PBS, cell cycle analysis was performed on the adherent cells. The S phase percentage is increased 2–2.5-fold in WT and S37A cells relative to CON. All experiments were performed at least three times with consistent and repeatable results.

were grown to confluence and cell cycle parameters were monitored. Pilot experiments revealed that the WT and S37A cells shed more cells into the medium than CON cells. To quantify this effect, cells that were two to three days after confluence were washed twice with PBS, and fresh medium was added. The medium was collected from the wells on the next day and the suspended cells were counted. The number of shed cells was markedly elevated in the WT and S37A cells, as compared with the CON cells (Fig. 4 A). In these experiments, shedding of the S37A cell pool was consistently higher than in the WT cell pool.

The hypothesis that a higher proliferative rate was responsible for the difference in cell shedding was tested by performing cell cycle analysis of these cells grown three days after confluence. This analysis demonstrated that the WT and S37A cells had a higher proportion of S phase and G_2 phase, and a lower percentage of G_0/G_1 phase, as compared with the CON cells (Fig. 4 B). This cell cycle profile is precisely what would be expected if the WT and S37A cells were proliferating more rapidly than the CON cells, and is consistent with other experiments in which the G_1/S checkpoint control regulates contact inhibition (Dietrich et al., 1997; Kato et al., 1997). Presumably, in the absence of additional space to attach to the culture dish, the newly formed cells are shed into the medium.

β-Catenin Attenuates the Radiation-induced G_1/S Cell Cycle Block

One important aspect of cell cycle regulation is cell cycle blockade after DNA damage. These blocks, which occur at the G_1/S and G_2/M transitions, presumably allow the cell to repair its DNA before the damage-induced errors become permanent (Weinert, 1998). We postulated that β-catenin overexpression might alter the DNA damage-induced late G_1 block of the cell cycle in the MDCK cells. The three cell pools were γ-irradiated with 0 or 5 Gy. Eight hours after irradiation, all of the cell pools show some G_1/S and G_2/M cell cycle blockade (Fig. 5). However, while CON had very few S-phase cells (5.96%), the WT and S37A cells retained a significant number of cells in S phase (15.26 and 14.99%). 24 h after irradiation, 25.2 and 21.4% of the WT and S37A cells, respectively, were in S phase, compared with 0.77% of CON cells. These data demonstrate that the radiation-induced G_1/S block is strongly attenuated by the overexpression of β-catenin and indicates that elevated β-catenin might lead to the accumulation of DNA damage and increased incidence of other mutations.

β-Catenin Expression Fluctuates throughout the Cell Cycle

The previously described block of G_1/S progression by APC in normal cells points to a role of endogenous β-catenin in the regulation of cell cycle progression in nontransformed cells (Baeg et al., 1995). Together, with our demonstration that even the modest elevations of β-catenin described in this study can regulate cell cycle progression,

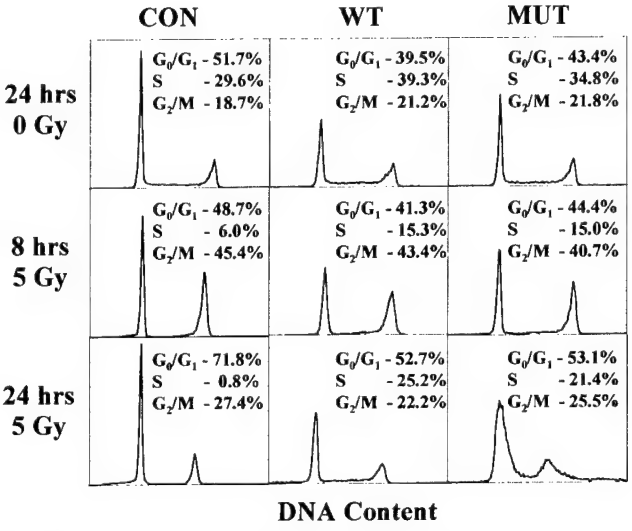

Figure 5. β-Catenin overexpression attenuates the γ-irradiation G_1 cell cycle block. Cells were γ-irradiated with 0 or 5 Gy. 8 and 24 h later, the cell cycle distribution was determined. The unirradiated cells all had a similar profile with the characteristic differences in G_0/G_1 and S phases (see Fig. 3). The S phase population of CON cells is significantly reduced at 8 h and absent at 24 h. At 24 h, the CON cells were blocked entirely in the G_0/G_1 or G_2 phases of the cell cycle. A slight decline in the proportion of WT and S37A cells in S phase occurs at 8 h after irradiation, but this is much less than that which occurs in CON cells. In contrast to CON cells, at 24 h the S phase proportions of the WT and S37A cells have partially recovered. All experiments were performed at least three times with consistent and repeatable results.

this led us to investigate its level of expression throughout the cell cycle. Preliminary experiments were performed with parental MDCK cells that were partially synchronized in early G_1 by serum starvation. Parallel wells of cells were collected at various time points after release from G_0 by the addition of serum to make whole cell or cytoplasmic lysates for analysis of β-catenin protein levels. Although total β-catenin protein did not vary appreciably during the cell cycle, cytoplasmic β-catenin levels increased significantly from G_1 to S phase (data not shown). The increase began in late G_1 and continued through S phase. These pilot experiments led us to examine this phenomenon in the A1N4 cell line, which is easily synchronized in early G_1 by the removal of EGF from the growth medium. Like MDCK cells, cytoplasmic levels of β-catenin protein increased in late G_1 and continued to rise in S phase (Fig. 6 A), whereas total cell β-catenin did not vary (data not shown). Densimetric scanning revealed a 23-fold increase in cytoplasmic levels from early G_1/G_0 to S phase (Fig. 6 B). As a control, the blot was reprobed for cyclin dependent kinase inhibitor, p27 (Fig. 6 A). As expected, variations in p27 were inversely related to β-catenin. To determine if this oscillation in cytoplasmic β-catenin led to fluctuations in β-catenin–LEF/TCF signaling, A1N4 cells were assayed for TOPFLASH activity after being synchronized in G_1 phase or G_2/M phase of the cell cycle. The level of β-catenin–LEF/TCF signaling corresponded with the levels of cytoplasmic β-catenin measured by Western blotting (Fig. 6 C). The elevation in signaling at G_2/M was greater than that induced by treatment with the proteosomal inhibitor, ALLN. These data indicate that oscillations in β-catenin signaling may be involved in the normal regulation of cell cycle progression.

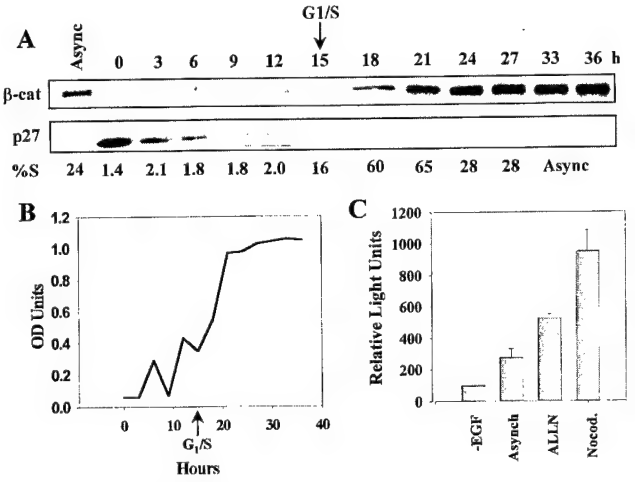

Figure 6. Cytoplasmic β-catenin oscillates during the cell cycle. A, A1N4 cells were synchronized in G_0/G_1 by EGF starvation. After releasing the cells into the cell cycle by the addition of EGF, cytoplasmic lysates were made every 3 h and assayed for β-catenin and p27 protein by immunoblotting. The distribution of cells in the cell cycle was determined at each time point by analyzing parallel cell cultures by flow cytometry. The percentage of S phase cells (%S) is provided. B, The level of expression was determined at each time point by densitometry and the results plotted against time after EGF addition. C, β-catenin–LEF/TCF signaling was measured in cells that were blocked in G_0/G_1 by EGF starvation (−EGF), growing asynchronously (Asynch), blocked near the S/G_2 transition by the proteosomal inhibitor ALLN, or blocked at G_2/M with nocodazole (Nocod). The results are expressed relative to the G_0/G_1 synchronized samples. Experiments represented in A and B were performed three times with consistent and repeatable results. Experiments represented in C were performed twice with consistent and repeatable results.

β-Catenin Promotes Colony Formation in Soft Agar

The ability of cells to proliferate in the absence of attachment to a solid substrate correlates well with the transformed, tumorigenic phenotype. To assess the oncogenic capacity of β-catenin in vitro, cells were suspended in 0.3% agar and allowed to grow for two weeks. The ability of the WT and S37A cells to form colonies in soft agar was clearly enhanced relative to the CON cells (Fig. 7, A–C). Although the CON cells do exhibit a background level of colony formation, expression of the β-catenin transgenes resulted in a 10–20-fold increase in the number of colonies and an obvious increase in colony size (Fig. 7 D). Multiple experiments did not demonstrate a significant difference between the WT and S37A cell pools. This is the first demonstration that full-length β-catenin, WT and S37A mutant, has transforming capacity.

β-Catenin Inhibits Anoikis

When nontransformed epithelial cells are deprived of attachment to an extracellular matrix for an extended period of time they undergo apoptosis (Frisch and Francis, 1994;

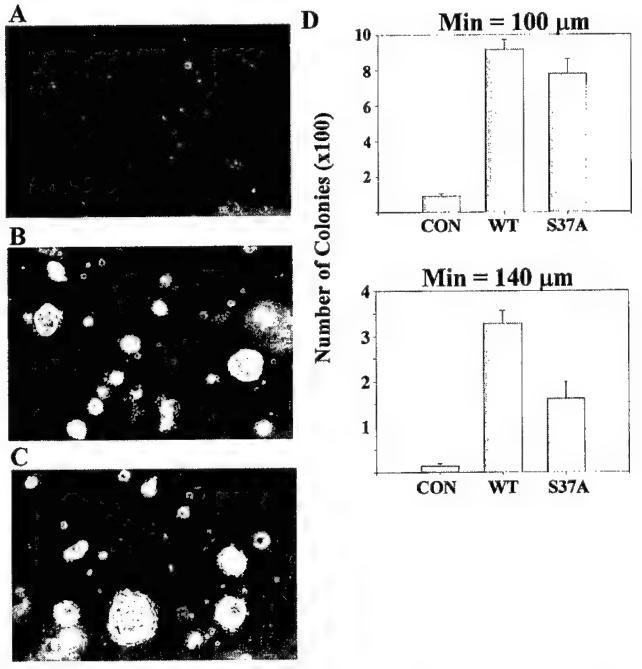

Figure 7. β-Catenin overexpression regulates soft agar colony formation. A–C, Phase-contrast photographs of colonies formed by the CON (A), WT (B), and S37A (C) cell pools after 14 d in soft agar. D, The number of colonies per 35-mm dish quantified by the Omnicon 3600 colony counter, using either 100 or 140 μm as the threshold for colony diameter. Experiments were repeated three times with consistent and repeatable results.

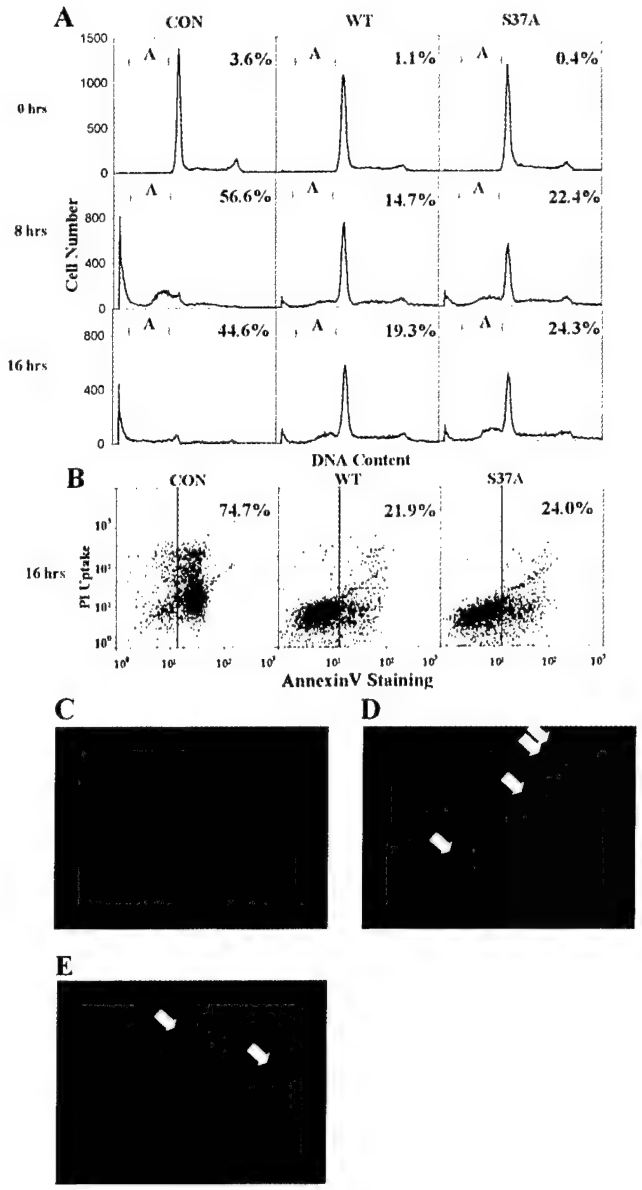

Figure 8. β-Catenin expression prevents anoikis. A, DNA/flow cytometric analysis of CON, WT, and S37A cells after incubation in suspension for 0, 8, or 16 h. The hypodiploid population corresponds to the apoptotic cells. The percentage in each panel represents the hypodiploid fraction. B, AnnexinV and propidium iodide staining of the same cells at 16 h also demonstrates a significant protection by β-catenin. C, Hoechst staining demonstrates nuclear morphology of CON cells before suspension. All nuclei look normal. WT and S37A cells looked similar. D, Hoechst staining of CON cells after 16 h in suspension. Most nuclei have a shrunken apoptotic morphology (arrows). E, S37A cells after 16 h in suspension. Most cells had the normal nuclear morphology, but a significant fraction (~25%) were shrunken apoptotic (arrows). Experiments represented in A and B were performed at least three times with consistent and repeatable results. Experiments represented in C–E were performed twice with consistent and repeatable results.

Frisch and Ruoslahti, 1997). This suspension-induced apoptosis has been termed anoikis. In the soft agar growth experiments, it appeared that most CON cells die when suspended in soft agar. However, the remaining cells did contribute to a background rate of colony formation. To investigate the possibility that β-catenin increases the colony-forming capacity of MDCK cells by preventing anoikis, cells were cultured on a cushion of 0.8% agar in normal growth medium, collected at eight hour intervals over a 24-h period, and assayed for apoptosis. Microscopic examination of the cells after 16- and 24-h incubations revealed that the majority of the WT and S37A cells were larger and more refractile to light than the CON cells (data not shown), suggesting that the CON cells were preferentially undergoing apoptosis. These preliminary results were confirmed by DNA/flow cytometry and AnnexinV staining of cells that had been kept in suspension for 0, 8, or 16 h (Fig. 8, A and B). Both methods showed that anoikis was significantly inhibited by β-catenin overexpression.

The results of further analysis of the flow cytometry and AnnexinV data for the percentage of hypodiploid and AnnexinV-positive cells, respectively, are compiled in Table I. The DNA/flow cytometry data revealed that the percentage of hypodiploid cells was markedly and consistently lower in the WT and S37A cells relative to the CON cells. However, these data significantly underestimate the percentage of apoptotic cells in the CON samples at the 16 h time point, as the disintegrating apoptotic cells were lost from the analysis. The AnnexinV assays appeared to retain these cells and probably give a more accurate estimate at 16 h.

As a third independent method of measuring apoptosis, nuclear morphology of cells before and after suspension was analyzed by Hoechst staining. In contrast to the nonsuspended cells, which all had normal nuclear morphology (Fig. 8 C), most of the suspended CON cells displayed characteristically shrunken apoptotic nuclei (Fig. 8 D). In contrast, the nuclei of the majority of WT and S37A cells displayed a normal morphology (Fig. 8 E). A fraction of the cells (~1/4) were apoptotic, which is consistent with the AnnexinV and flow cytometry results. Interestingly, a minority of CON cells were found to be associated with clumps of five or more cells. Most of these cells displayed normal nuclear morphology. This was a clear demonstration that cell–cell adhesion can prevent apoptosis induced by suspension, and this probably caused us to underestimate the percentage of apoptosis among the suspended CON cells by the AnnexinV and flow cytometric methodologies.

These data demonstrate that β-catenin overexpression may promote soft agar colony formation of MDCK cells by the promotion of cell cycle progression and the inhibition of anoikis.

Table I. β-Catenin Prevents Anoikis, as Measured by DNA/Flow Cytometry and AnnexinV Labeling

	Hypodiploid cells			AnnexinV positive		
	0 h	8 h	16 h	0 h	8 h	16 h
		%			%	
CON	3.6	**56.6**	44.6	1.7	50.4	**74.7**
WT	1.1	**14.7**	19.1	2.4	18.0	**21.9**
MUT	0.4	**22.4**	24.3	0.7	31.0	**24.0**

The percentage of apoptotic cells in the three cell pools after different periods of suspension, as measured by flow cytometry (hypodiploid) or AnnexinV labeling (AnnexinV positive). The percentages in **bold** demonstrate the most notable effects.

Discussion

It is suspected that the cadherin-associated protein β-catenin promotes the process of carcinogenesis (Peifer, 1997). The data that support this hypothesis include the following observations: it associates with and is downregulated by the tumor suppressor APC; it transduces (at least partly) the oncogenic Wnt growth factor signal to the nucleus; it is mutated in a significant number of human cancers; and, overexpression of an NH_2 terminally truncated form of β-catenin in the epidermis of transgenic mice produced well-differentiated hair tumors (Rubinfeld et al., 1993; Su et al., 1993; Cadigan and Nusse, 1997; Ilyas et al., 1997; Fukuchi et al., 1998; Gat et al., 1998; Miyoshi et al., 1998; Palacios and Gamallo, 1998; Voeller et al., 1998). However, no studies provide direct evidence for the transforming potential of full-length β-catenin. In addition, no investigations have addressed the question of which cellular processes β-catenin may regulate to effect cellular transformation.

β-Catenin Transforms the Epithelial MDCK Cell Line

This report characterizes phenotypic alterations that result from β-catenin overexpression in a nontransformed epithelial cell line. Effects are seen in the regulation of three important cellular activities/properties: proliferation, apoptosis, and morphology. It demonstrates that modest β-catenin overexpression significantly enhances the ability of these cells to proliferate, especially in situations that would normally inhibit the cell cycle at the G_1/S transition. Most striking is the demonstration that it promotes growth in soft agar, a phenotype closely correlated with tumorigenicity. Most nontransformed cells require adhesion through integrin receptors to extracellular matrix components to transit through the G_1 phase of the cell cycle (Mehta et al., 1986; Polyak et al., 1994). In addition, suspension of normal, attachment-dependent cells blocks them late in G_1 phase.

β-Catenin overexpression also resulted in increased proliferation of cells at high cell density. The mechanism by which high cell density inhibits proliferation is unknown, but also involves a block in late G_1. The presence of cell–cell adhesion, the reduction of cell-substrate adhesion, and the depletion of growth factors have all been implicated (Chen et al., 1997). β-Catenin's dual activities as a regulator of cadherin-mediated cell–cell adhesion and as the transducer of a mitogenic signal implicate it in this regulatory process. Both cadherin and α-catenin can inhibit β-catenin signaling in other experimental systems (Fagotto et al., 1996; Simcha et al., 1998). Together, with the results of the present study, these data support the hypothesis that cell–cell adhesion promotes the formation of cadherin/β-catenin/α-catenin complexes and that these complexes negatively regulate β-catenin signaling, which discourages cell cycle progression. However, the fact that proliferation is reduced at high cell density, as compared with sparsely plated cells, even in the WT and S37A cells, suggests that other mechanisms are also involved (for example, cell shape; Chen et al., 1997).

The cell cycle analyses and growth curves in this study demonstrate that β-catenin overexpression can significantly alter the proliferative rate of these cells. The distribution of the WT and S37A cells is weighted heavily toward S phase and away from G_1. When considered along with the other cell cycle data, it appears that β-catenin overexpression expedites the G_1/S transition in MDCK cells. The easing of the barrier to G_1/S transition manifests as a difference in cell growth on plastic, as growth curves of the β-catenin overexpressing cells diverged significantly from the control cells.

β-Catenin overexpression also has a notable effect on cell morphology. The MDCK cell line is a nontransformed epithelial line that has very strong intercellular adhesion and extends cell membrane extensions only to a limited degree. β-Catenin overexpression converts MDCKs into a more mesenchymal cell type (Barth et al., 1997; and the present study). At low density, cell–cell adhesion is reduced and the cells take on a more spindly, stretched shape. This change in morphology is reminiscent of an epithelial to mesenchymal transition (EMT; Huber et al., 1996). EMTs are developmentally important cellular conversions, especially during gastrulation, the point in development at which β-catenin knockout mouse embryos are aborted. Also, an EMT has been suggested to underlie the progression from benign tumor to metastatic carcinoma (Sommers et al., 1991; Birchmeier et al., 1996). Indeed, it previously has been suggested that β-catenin signaling may regulate this process (Sommers et al., 1994; Huber et al., 1996).

The absence of anoikis is another characteristic of transformed cells. The present study and others have shown that MDCK cells are very dependent on attachment to the extracellular matrix for survival (Frisch and Francis, 1994; Frisch et al., 1996a,b). After 16 h in suspension, the majority of CON cells were apoptotic, as measured by three independent methods. The expression of the WT and S37A β-catenin transgenes markedly retards this process, allowing ~75% of the single cells to survive. This is a vigorous inhibition of anoikis. Taken together, the proliferation, anoikis, and morphology data demonstrate that these cells are clearly transformed by β-catenin.

These in vitro results suggest that overexpression of full-length β-catenin should promote tumorigenesis in vivo. Two separate studies have demonstrated the effect of tissue-specific overexpression of an NH_2 terminally truncated form of β-catenin. Expression of the truncated form of β-catenin in the epidermis of transgenic mice by Gat et al. (1998) resulted in the formation of two types of hair follicle-related tumors. Taken together with the present study, these results strongly suggest that full-length forms of β-catenin are important mediators of oncogenesis in vivo. Interestingly, a study by Wong et al. (1998), in which an NH_2 terminally truncated form of β-catenin was overexpressed in the intestinal epithelium of transgenic mice, produced conflicting results. Proliferation of the intestinal epithelial cells in these animals was stimulated 1.5–3-fold, in accordance with the results of the present study. However, the elevated proliferation rate was balanced by an increase in apoptosis, the net result being no change in intestinal villus height. To explain the discrepancy between these results and our own, we suggest that β-catenin overexpression can protect cells only from certain apoptotic signals. It is possible that the compensatory mechanism by which the authors suggested that the transgenic mice might have maintained their cell census in the face of in-

creased proliferation is mediated through the stimulation of β-catenin–insensitive apoptosis. It is also possible that full-length β-catenin has signaling capacities that are lost when its NH_2 terminus is removed.

The results presented in the present study also differ from those published previously by Young et al. (1998). They reported that overexpression of the Wnt-1 growth factor transformed Rat-1 fibroblasts while expression of the S37A mutant form of β-catenin we described previously had no effect. Two differences between the two studies may explain the conflicting results. First, the morphological effects we describe may only be detectable in an epithelial cell type. Second, the studies of Young et al. (1998) were carried out without serum, whereas the present ones were done with serum. It is possible that Wnt-1 activates parallel signaling pathways (in addition to β-catenin signaling) that may circumvent the need for serum to stimulate proliferation. β-Catenin's position lower in the pathway may preclude the activation of such parallel pathways and, therefore, it is unable to stimulate proliferation of Rat-1 fibroblasts in the absence of serum.

β-Catenin Attenuates the Cell's Response to γ-Irradiation

The cell cycle blocks that characterize the response of cells to DNA damage are important for the maintenance of genomic integrity. To prevent the permanent incorporation of mutations induced by various DNA damaging stimuli, the cell cycle can pause at the G_1/S and the G_2/M transitions (Weinert, 1998). During these delays, the cell assesses the damage to its DNA and either repairs the damage or destroys itself. Premature reentry into the cell cycle may result in the accumulation of mutations to oncogenes and tumor suppressor genes, which would increase the likelihood of cellular transformation and cancer. The data from this study suggest that β-catenin overexpression may result in the premature reentry of cells into the cell cycle after γ-irradiation–induced DNA damage, and thereby promote the accumulation of oncogene mutations and carcinogenesis.

β-Catenin Overexpression Inhibits Anoikis

An association between apoptosis and the APC/β-catenin axis has been suggested previously. Reexpression of the APC gene in a tumor cell line that lacks WT APC resulted in the induction of apoptosis within 24 h (Morin et al., 1996). Since one of the functions of APC is to downregulate β-catenin, it is possible that β-catenin itself is a regulator of apoptosis. Our demonstration that β-catenin alone significantly protects cells from anoikis strongly implies that it can be a potent inhibitor of apoptosis. Also, during the process of apoptosis, caspase-3 can cleave β-catenin protein (Brancolini et al., 1997). One purpose of this cleavage may be to destroy the antiapoptotic β-catenin signal within the cell and thereby hasten the completion of the apoptotic process. The caspase-mediated cleavage of focal adhesion kinase (FAK) is thought to function in this manner (Wen et al., 1997).

It has been postulated that the induction of apoptosis by the loss of appropriate extracellular matrix attachment (i.e., anoikis) is a means of protecting the organism from improper cell growth (Frisch and Ruoslahti, 1997). Anoikis is prevented by integrin-mediated signaling. Several enzymes have been implicated as being downstream of integrins in this signal transduction pathway. These include FAK, phosphoinositide-3-kinase, protein kinase B/Akt, and integrin-linked kinase (ILK; Clark and Brugge, 1995; Giancotti, 1997; Wu et al., 1998). The present report suggests that β-catenin may also lie downstream of integrins. Several integrin-stimulated signaling pathways might lead to the induction of β-catenin signaling. One possible connection between integrins and β-catenin is the integrin-activated, antiapoptotic kinase PKB/Akt. PKB is known to inhibit the activity of glycogen synthase kinase 3-β, a serine kinase that functions directly to reduce β-catenin protein and signaling (Siegfried et al., 1992; Cook et al., 1996; Cadigan and Nusse, 1997). It is possible that the result of these two inhibitory interactions is that activation of PKB by integrin signaling functions to positively activate β-catenin signaling.

The data presented in this report describing the effects of β-catenin overexpression are similar to previous reports describing the effects of ILK (Novak et al., 1998; Wu et al., 1998). ILK is a 59-kD serine kinase that was first described as a β_1-integrin-associated kinase. ILK overexpression causes cells to undergo an EMT and promotes their growth in soft agar. This is associated with an increase in LEF-1 protein levels. As a result of increased LEF-1, β-catenin becomes completely localized to the nucleus and β-cat-LEF/TCF signaling increases significantly. In addition, loss of cell attachment to the underlying ECM was shown to result in a dramatic reduction in LEF protein. In a separate study, ILK directly phosphorylated and inhibited the activity of GSK-3β. This may constitute another mechanism by which integrin signaling may result in increased β-catenin–LEF/TCF signaling.

Anoikis results from the interruption of integrin-mediated signaling (Frisch and Ruoslahti, 1997). In addition to ILK, the integrin-associated nonreceptor tyrosine kinase FAK may also be involved in the transduction of these signals because FAK signaling suppresses p53-dependent apoptosis (Ilic et al., 1998). Ilic et al. (1998) also demonstrated that an atypical protein kinase C isoform (PKCλ/ι) is required for this p53-dependent apoptotic pathway, since inhibition with both chemical PKC inhibitors and a dominant–negative construct protect FAK-defective cells from apoptosis. Previously, we reported that an atypical PKC isoform was involved in regulating β-catenin degradation (Orford et al., 1997). Inhibiting atypical PKC activity using the same chemical PKC inhibitors used by Ilic et al. (1998) resulted in the inhibition of the ubiquitination and degradation of β-catenin. In addition, treatment of cells with these PKC inhibitors increases β-catenin–LEF/TCF signaling (unpublished results). Taken together with the present study, it is possible that the inhibition of PKCλ/ι or another atypical PKC may increase β-catenin stability and signaling, leading to the suppression of p53-mediated apoptosis (Fig. 9 A).

β-Catenin Oscillations during the Cell Cycle May Regulate Normal Cellular Proliferation

The c-myc promoter is also regulated by the APC/β-cate-

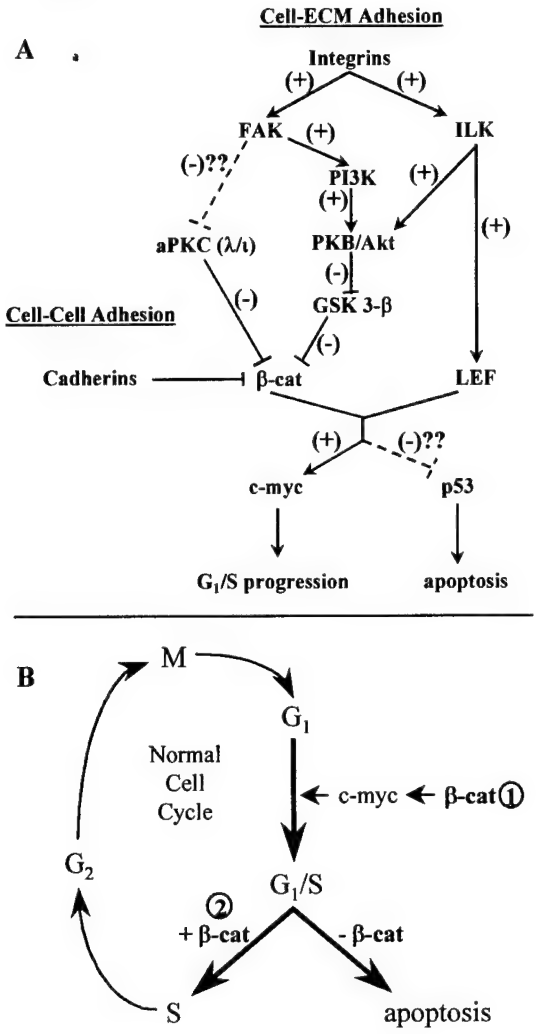

Figure 9. Hypothetical signaling pathways by which β-catenin might integrate cell adhesion, cell cycle, and apoptosis. A, The individual regulatory relationships depicted by unbroken arrows and blockades have been demonstrated in various published reports. However, these signaling pathways have never been demonstrated in their entirety within a single experimental system. The broken blockades are hypothetical regulatory events suggested in the present report. Integrin-activated FAK activity may regulate β-catenin signaling by two different pathways. In both cases, two sequential negative regulatory interactions downstream of FAK may result in the activation of β-catenin signaling. By a parallel pathway, ILK can regulate the activities of PKB and GSK-3β, as well as upregulate the expression of the transcription factor LEF-1. Together, β-catenin and LEF-1 might stimulate the G_1/S transition in the cell cycle (possibly via c-myc) and inhibit p53-mediated apoptosis. The inhibition of apoptosis may be through direct modulation of p53 action or through a parallel antiapoptotic pathway. The role of p53 in β-catenin–mediated signaling is speculative. B, β-catenin may regulate the cell cycle by two separate mechanisms: 1, β-catenin can stimulate the expression of c-myc, which is a strong stimulator of cell cycle progression; 2, the G_1/S transition represents an important decision-point for the cell. It is known that this transition requires the presence of survival factors. In their absence, the cell chooses apoptosis over proliferation. β-Catenin may regulate the G_1/S transition as a survival factor functioning to permit cell cycle progression by preventing apoptosis.

nin signaling pathway (He et al., 1998). The upregulation of c-myc by β-catenin may constitute one mechanistic link between β-catenin and tumor formation. c-myc is potent oncogene that regulates cell cycle progression. However, c-myc overexpression cannot induce cellular transformation on its own. In fact, when overexpressed alone, c-myc markedly increases the susceptibility of cells to apoptosis (Desbarats et al., 1996; Steiner et al., 1996; Thompson, 1998). To transform cells, c-myc requires an accompanying survival signal to prevent cells from undergoing apoptosis. Advancement through the G_1 phase of the cell cycle can result in either progression into S phase or apoptosis, depending on the presence or absence of certain survival signals, for example, IGF-1 (Evan et al., 1995). In addition to stimulating c-myc, β-catenin may transduce the requisite antiapoptotic signal that would permit cell cycle progression. The increase of cytoplasmic β-catenin protein before S phase during the cell cycle may serve this purpose in normal cells (Fig. 6). Additionally, β-catenin would protect against anoikis if overexpressed in epithelial cells.

Our data do not demonstrate any reproducible phenotypic difference between the WT and S37A expressing cells, except in the measurement of protein expression and in cell shedding at confluence. It is important to note that in both the WT and S37A cell pools, the level of cytoplasmic β-catenin protein and β-catenin–LEF/TCF signaling is elevated relative to the CON cells. This implies that a modest increase of cytoplasmic β-catenin can result in significant changes in signaling and cellular transformation and that overexpression of the wild-type gene alone is sufficient. This may also explain how the relatively small increase in endogenous cytoplasmic β-catenin that occurs before the onset of S phase may regulate the G_1/S transition in the normal cell cycle (Fig. 9 B). However, it is interesting to note that the increase in signaling above CON levels and the difference between the WT and S37A cells are relatively small when compared with other published results (Morin et al., 1997; Porfiri et al., 1997; Young et al., 1998). It is possible that the fact that this study was performed with cells that stably express a constitutively active transgene is responsible for both phenomena. We believe that the very high levels of β-catenin expression and signaling that can be achieved in nontransformed cells by transient transfection is not conducive to their survival and propagation. If true, selection pressures against very high expression would: result in the production of stable cells expressing only moderately elevated β-catenin protein and signaling; and, limit the extent to which the S37A mutation could stimulate signaling above WT β-catenin. In addition, some studies have used different β-catenin mutants, which may be more active.

It is plausible that some of the phenotypic alterations induced by β-catenin overexpression could be the result of altered cadherin function and independent of β-catenin signaling. However, the fact that these cells display strong intercellular adhesion at high density and retain the ability to generate tight junctions (as measured by electrical resistance across the monolayer in culture) demonstrates that E-cadherin function remains intact.

The APC/β-catenin signaling pathway has been implicated in a large number of epithelial cancers (Munemitsu et al., 1995; Inomata et al., 1996; Ilyas et al., 1997; Korinek

et al., 1997; Mareel et al., 1997; Morin et al., 1997; Peifer, 1997; Rubinfeld et al., 1997; Palacios and Gamallo, 1998; Voeller et al., 1998). In most cases, mutations in either APC or β-catenin result in stabilization of β-catenin protein and elevated β-catenin–LEF/TCF signaling. However, it is not clear what role this pathway has in normal cells. In this study, we demonstrate that β-catenin is a potent oncogene. All of the major phenomena that characterize cellular transformation, that is, soft agar growth, altered morphology, inhibition of apoptosis, and stimulation of cell cycle progression, can be induced by the modest overexpression of β-catenin in a nontransformed epithelial cell line. This clearly indicates that β-catenin can play a direct role in the process of carcinogenesis and that a major component of APC function is its downregulation. These data suggest that, as an early event in the progression of colorectal cancer, activation of β-catenin signaling promotes adenoma formation by promoting proliferation and survival of epithelial cells in the abnormal tissue architecture of a tumor mass. In addition, it may also promote the accumulation of mutations and cancer progression by attenuating the DNA damage-induced G_1 cell cycle block.

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The Ubiquitin-Proteasome Pathway and Serine Kinase Activity Modulate Adenomatous Polyposis Coli Protein-mediated Regulation of β-Catenin-Lymphocyte Enhancer-binding Factor Signaling*

(Received for publication, January 21, 1999)

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The tumor suppressor function of the adenomatous polyposis coli protein (APC) depends, in part, on its ability to bind and regulate the multifunctional protein, β-catenin. β-Catenin binds the high mobility group box transcription factors, lymphocyte enhancer-binding factor (LEF) and T-cell factor, to directly regulate gene transcription. Using LEF reporter assays we find that APC-mediated down-regulation of β-catenin-LEF signaling is reversed by proteasomal inhibitors in a dose-dependent manner. APC down-regulates signaling induced by wild type β-catenin but not by the non-ubiquitinatable S37A mutant, β-catenin. Bisindoylmaleimide-type protein kinase C inhibitors, which prevent β-catenin ubiquitination, decrease the ability of APC to down-regulate β-catenin-LEF signaling. All these effects on LEF signaling are paralleled by changes in β-catenin protein levels. Lithium, an inhibitor of glycogen synthase kinase-3β, does not alter the ability of APC to down-regulate β-catenin protein and β-catenin-LEF signaling in the colon cancer cells that were tested. These results point to a role for β-catenin ubiquitination, proteasomal degradation, and potentially a serine kinase other than glycogen synthase kinase-3β in the tumor-suppressive actions of APC.

Mutations in the tumor suppressor adenomatous polyposis coli (APC)[1] gene are responsible for tumors that arise in both familial adenomatous polyposis and sporadic colon cancers (1–7). APC mutations are almost always truncating, giving rise to proteins lacking C termini (6, 8, 9). Efforts to understand how these mutations contribute to cancer have focused on the ability of APC to bind and subsequently down-regulate the cytoplasmic levels of β-catenin (10–13).

β-Catenin is a multifunctional protein that participates in cadherin-mediated cell-cell adhesion and in transduction of the Wnt growth factor signal that regulates development (14, 15). Activation of the Wnt growth factor signaling cascade results in the inhibition of the serine/threonine kinase, GSK-3β, and in response, β-catenin accumulates in the cytoplasm (16–18). At elevated cytoplasmic levels, β-catenin translocates to the nucleus, interacts with the high mobility group box transcriptional activator lymphocyte enhancer-binding factor (LEF)/T-cell factor, and directly regulates gene expression (19–22). Mutations that stabilize β-catenin protein are likely to be oncogenic, although this has not been proven directly (23).

The mechanism of APC-mediated β-catenin regulation is unknown. Recently, β-catenin was shown to be regulated at the level of protein stability via proteasomal degradation (24, 25). Proteins targeted for degradation by the ubiquitin-proteasome system are first tagged with multiple copies of the small protein ubiquitin by highly regulated ubiquitination machinery (27). Polyubiquitinated proteins are recognized and rapidly degraded by the proteasome, a large multisubunit proteolytic complex. Proteasomal degradation plays a critical role in the rapid elimination of many important regulatory proteins, *e.g.* cyclins and transcriptional activators like NFκB-IκB (28). Proteins regulated via proteasomal degradation can be specifically studied using the well characterized proteasome-specific peptidyl-aldehyde inhibitors (29, 30).

APC-mediated tumorigenesis might depend, in part, on its ability to regulate β-catenin signaling (26). In this report, we show that the ubiquitin-proteasome pathway and the activity of a serine kinase other than GSK-3β modulate APC-mediated regulation of β-catenin-LEF signaling.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells—ALLN, ALLM, lactacystin-β lactone, and MG-132 were purchased from Calbiochem. GF-109203X was purchased from Roche Molecular Biochemicals. Ro31-8220 was a gift from Dr. Robert Glazer. The monoclonal anti-β-catenin antibody (Clone 14) and the anti-FLAG™ antibody were purchased from Transduction Laboratories, Lexington, KY and Eastman Kodak Co., respectively. Affinity-purified rabbit polyclonal anti-APC2 and anti-APC3 antibodies (12) were generously provided by Dr. Paul Polakis (Onyx Pharmaceuticals). Affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies were purchased from Kirkegaard and Perry Laboratories. The SW480 and CACO-2 colon cancer cell lines were acquired from the ATCC and maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 1% penicillin/streptomycin.

Transfections and LEF-Luciferase Reporter Assays—Cells were seeded in 12-well plates at 1×10^5 cells/well. The following day cells were transiently transfected with 1 μg of APC constructs and 0.4 μg of the LEF reporter, pTOPFLASH (optimal motif), or pFOPFLASH (mutant motif) (31), and 0.008 μg of pCMV-*Renilla* luciferase (Promega) per well, using LipofectAMINE-Plus reagent according to the manufacturer's instructions (Life Technologies, Inc.) for 5 h. In experiments designed to monitor the effect of APC on β-catenin protein, 0.3 μg of FLAG-tagged WT or S37A β-catenin (25) was cotransfected with 0.6 μg of empty vector or APC constructs. This approach facilitated analysis of only the transfected cells, using anti-FLAG antibodies.

Cells were treated with indicated levels of the inhibitors for 12–24 h. Luciferase activity was monitored using the dual luciferase assay sys-

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[1] The abbreviations used are: APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; LEF, lymphocyte enhancer-binding factor; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ALLM, *N*-acetyl-Leu-Leu-methional; WT, wild type; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DAG, diacylglycerol; NKκB, nuclear factor κB; IκB, inhibitor of NFκB.

tem (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency and potential toxicity of treatments using the constitutively expressed pCMV-*Renilla* luciferase. The specificity of APC-mediated effects on LEF reporters was confirmed using pFOPFLASH, which harbors mutated LEF binding sites (31), and an unrelated AP-1 reporter (32).

Immunological Procedures—Double immunofluorescent staining for APC and β-catenin was performed according to Munemitsu *et al.* (11, 40). In experiments where FLAG-tagged β-catenin was cotransfected with APC, anti-FLAG™ antibodies (Kodak) were used to detect the exogenous β-catenin.

RESULTS AND DISCUSSION

APC-mediated Down-regulation of β-Catenin-LEF Signaling Is Reversed by Proteasomal Inhibitors—In the SW480 colon cancer cell line, which produces only a mutant APC protein containing amino acids 1–1337 of the complete 2843-amino acid sequence, overexpression of WT APC or deletion construct APC 25 (amino acids 1342–2075), but not APC 3 (amino acids 2130–2843) (Fig. 1*A*), can effect a posttranslational down-regulation of β-catenin (11, 26). We tested the hypothesis that APC effects the down-regulation of β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation. SW480 cells were transiently transfected with various APC deletion constructs (Fig. 1*A*) and treated with proteasomal inhibitors, and β-catenin-LEF signaling was assayed using LEF reporters (31). Fig. 1*B* shows that the APC-mediated down-regulation of β-catenin-LEF signaling is reversed by a panel of proteasomal inhibitors including ALLN, lactacystin-β lactone, and MG-132, but not Me_2SO (vehicle) or ALLM (calpain inhibitor II), that effectively inhibits calpain proteases but has a 100-fold lower potency as a proteasomal inhibitor. The specificity of APC-mediated effects on LEF reporters was confirmed using pFOPFLASH, which harbors mutated LEF binding sites, and an unrelated AP-1 reporter, neither of which was influenced by APC (31, 32). The proteasomal inhibitor ALLN reverses the APC- mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner (Fig. 1*C*). The effects of APC 25 can be completely reversed by the proteasomal inhibitor ALLN, and the effects of WT APC can be restored to 50–60% of control values. However, the full-length WT APC construct, and not the APC 25 deletion construct, was used for all immunostaining experiments because it was more physiologically relevant (incorporating all the functional domains). SW480 cells were transfected with empty vector or WT APC and were treated with Me_2SO (vehicle) or the proteasomal inhibitors ALLN or lactacystin-β lactone. Double immunofluorescent staining for APC (Fig. 2, *A*, *C*, and *E*) and β-catenin (Fig. 2, *B*, *D*, and *F*) shows that the APC induced reduction in β-catenin protein (Fig. 2, *A* and *B*) is reversed by proteasomal inhibitors ALLN (Fig. 2, *C* and *D*) and lactacystin-β lactone (Fig. 2,*E* and *F*).

APC Down-regulates WT β-Catenin but Not the Non-ubiquitinatable S37A Mutant Form of β-Catenin-induced LEF Signaling—Mutation of a single serine residue (S37A) within the ubiquitination-targeting sequence prevents β-catenin ubiquitination (25). Serine mutations in the ubiquitin-targeting sequence of β-catenin occur in a number of different cancers (33–38). At least one of these, S37A, is a stabilizing mutation that renders β-catenin resistant to ubiquitination (25). If indeed APC regulates β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation, then it should not be able to down-regulate the non-ubiquitinatable S37A mutant β-catenin protein or the LEF signaling induced by this stable form of β-catenin. To test this hypothesis, vector, FLAG-tagged WT, or S37A mutant β-catenin constructs were cotransfected with vector or WT APC and the LEF reporters into SW480 cells. β-Catenin-LEF signaling was monitored by assaying LEF reporter activity. Overexpression of both WT and S37A mutant forms of β-catenin increased the basal LEF reporter activity by about 30%, even against the background of high levels of en-

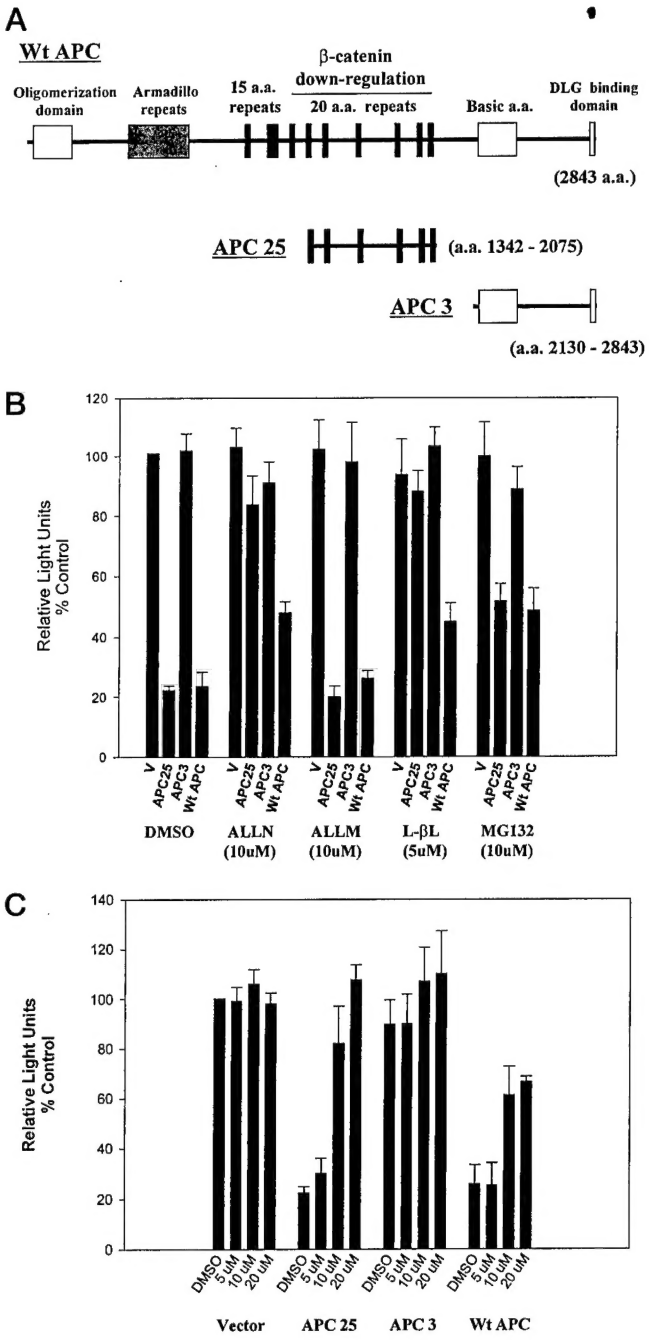

FIG. 1. *A*, the structure of WT APC and APC deletion constructs (26); *B*, APC-mediated down-regulation of β-catenin- LEF signaling is reversed by proteasomal inhibitors. SW480 cells were transiently transfected with various APC constructs, using LipofectAMINE-Plus reagent (Life Technologies, Inc.). 12 h posttransfection, the cells were treated with proteasomal inhibitors ALLN, lactacystin-β lactone, and MG-132 or with Me_2SO (*DMSO*, vehicle) and ALLM (calpain inhibitor II) for 12 h. β-Catenin-LEF signaling was assayed using the LEF reporters pTOPFLASH (and pFOPFLASH; data not shown) (31). Raw data were normalized for transfection efficiency and potential toxicity of treatments, using pCMV-*Renilla* luciferase and the dual luciferase assay system (Promega). The experiment was repeated at least three times, with each treatment repeated in triplicate. *Error bars* represent S.D. *C*, APC-mediated down-regulation of β-catenin-LEF signaling is reversed by the proteasomal inhibitor, ALLN, in a dose-dependent manner. The transfections were performed as described in *B* and were followed by treatment with the various doses (μM) of the proteasomal inhibitor, ALLN. *a.a.*, amino acid(s); *DLG*, Discs Large protein.

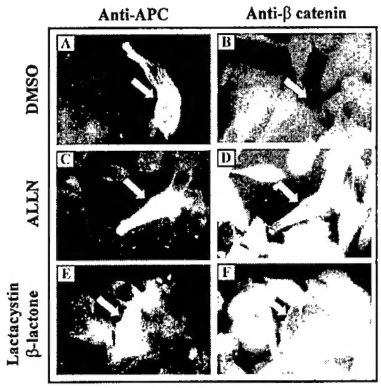

FIG. 2. **APC-mediated down-regulation of β-catenin protein is reversed by proteasomal inhibitors.** SW480 cells were transfected with WT APC and treated with Me_2SO (*DMSO*, *A* and *B*), 10 μM ALLN (*C* and *D*), or 5 μM lactacystin-β lactone (*E* and *F*). Double immunofluorescent staining for APC (*A*, *C*, and *E*) and β-catenin (*B*, *D*, and *F*) was performed according to Munemitsu *et al.* (11, 40).

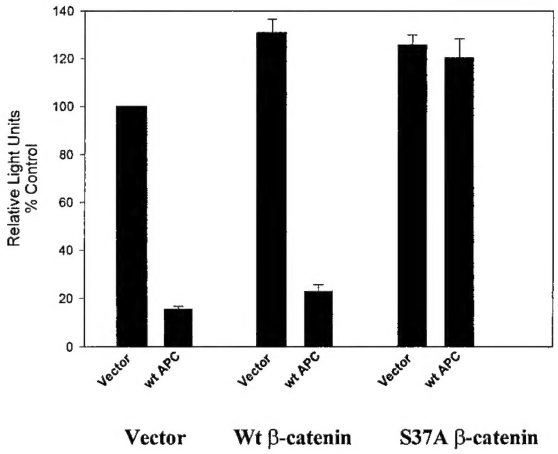

FIG. 3. **APC down-regulates LEF signaling induced by WT β-catenin but not by the non-ubiquitinatable S37A mutant β-catenin.** SW480 cells were transfected with empty vector or FLAG-tagged WT β-catenin or FLAG-tagged S37A β-catenin and empty vector or WT APC constructs, LEF reporters, and pCMV-*Renilla* luciferase. 24 h posttransfection, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

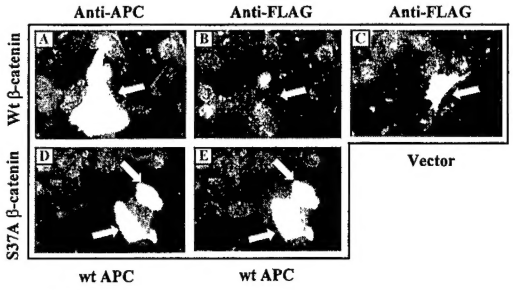

FIG. 4. **APC down-regulates WT β-catenin but not the non-ubiquitinatable S37A mutant β-catenin protein.** SW480 cells were transfected with FLAG-tagged WT β-catenin (*A*, *B*, and *C*) or FLAG-tagged S37A β-catenin (*D* and *E*) and WT APC constructs (*A*, *B*, *D*, and *E*) or empty vector (*C*). Double immunofluorescent staining for APC (*A* and *D*) and β-catenin (*B*, *C*, and *E*) were performed according to Munemitsu *et al.* (11, 40), except that the tranfected FLAG-tagged β-catenin was detected using anti-FLAG antibodies (Kodak).

dogenous β-catenin and β-catenin-LEF signaling in the SW480 cells. S37A β-catenin is more stable than WT β-catenin (in cells that actively degrade β-catenin, *e.g.* SKBR3 cells), but both forms increased LEF signaling by comparable levels in SW480 cells (which lack the ability to degrade β-catenin). Fig. 3 shows that APC down-regulates LEF signaling induced by WT β-catenin but not by the S37A mutant β-catenin. The ability of APC to down-regulate the cotransfected FLAG-tagged WT β-catenin and the S37A β-catenin protein levels was examined by double immunofluorescent staining using anti-APC antibodies and anti-FLAG antibodies (Kodak) (40). By double immunofluorescent staining for both the FLAG epitope and APC, we were able to monitor effects of APC specifically on the coexpressed forms of β-catenin. Fig. 4*A* (anti-APC) and Fig. 4*B* (anti-FLAG) show that WT APC effectively down-regulates WT β-catenin. Fig. 4*C* (anti-FLAG) shows that in concurrent transfections with empty vector and FLAG-tagged WT β-catenin, the FLAG-tagged WT β-catenin is expressed and the anti-FLAG antibody efficiently detects it. Fig. 4, *D* and *E* shows that APC does not down-regulate the S37A mutant β-catenin protein. These findings complement the observations of Munemitsu *et al.* (41) and Li *et al.* (42) that APC associates with but does not down-regulate β-catenin with an N-terminal deletion.

The Bisindoylmaleimide-type PKC Inhibitor GF-109203X Decreases the Ability of APC to Down-regulate LEF Signaling in a Dose-dependent Manner—PKC activity is required for Wnt-1 growth factor signaling to inhibit GSK-3β activity (18). TPA-induced down-regulation of diacylglycerol (DAG)-dependent PKCs prevents Wnt from inhibiting GSK-3β (18). However, our earlier studies demonstrate that neither the PKC inhibitor calphostin C nor TPA-induced down-regulation of PKCs stabilizes β-catenin (25). In contrast, the bisindoylmaleimide-type PKC inhibitor GF-109203X causes a dramatic accumulation of β-catenin in the cytoplasm (25). The bisindoylmaleimides inhibit both DAG-dependent and -independent PKC isoforms by competing with ATP for binding to the kinase, whereas calphostin C and long term TPA treatment inhibit only DAG-dependent PKC activities. The inhibitor profile implicates DAG-independent, atypical PKC activity in regulating β-catenin stability. These kinase(s) may offer a level of regulation distinct from the DAG-dependent PKC isoforms that regulate Wnt-dependent and GSK-3β-mediated β-catenin signaling (25).

The bisindoylmaleimide-type PKC inhibitor GF-109203X prevents β-catenin ubiquitination but does not inhibit GSK-3β (25). We tested the hypothesis that GF-109203X will inhibit the ability of APC to regulate β-catenin-LEF signaling. Fig. 5 shows that the PKC inhibitor GF-109203X decreases the ability of APC to down-regulate LEF signaling in a dose-dependent manner in SW480 cells. The changes in β-catenin-LEF signaling are paralleled by changes in β-catenin protein (Fig. 6). Similar results were obtained with another bisindoylmaleimide-type PKC inhibitor Ro31-8220 (data not shown).

Lithium (Li^+) Does Not Inhibit the Ability of APC to Down-regulate β-Catenin-LEF Signaling—Physiologically effective concentrations of Li^+ specifically and reversibly inhibit GSK-3β activity *in vitro* and *in vivo* and can mimic the effects of Wnt signaling on β-catenin in mammalian cells (43–46). Treatment of breast cancer cell lines with lithium results in the accumulation of the cytoplasmic signaling pool of β-catenin (25). Axin, the recently described product of the mouse *Fused* locus, forms a complex with GSK-3β, β-catenin, and APC (47). Axin promotes GSK-3β-dependent phosphorylation of β-catenin and may therefore help target β-catenin for degradation (48). However, overexpression of Axin inhibits β-catenin-LEF signaling in SW480 colon cancer cells in the absence of functional, WT APC. It is not known if APC promotes GSK-3β-dependent phosphorylation of β-catenin. Rubinfeld *et al.* (49) have shown that the APC protein is phosphorylated by GSK-3β *in vitro* and suggest that this phosphorylation event is linked to β-catenin turnover. It has also been suggested that APC and Axin may regulate the degradation of β-catenin by different mechanisms (50).

We tested the hypothesis that Li^+ can inhibit the ability of

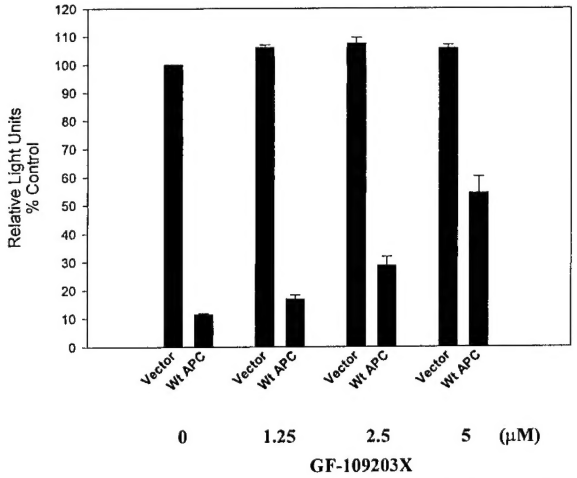

FIG. 5. **The bisindoylmaleimide-type PKC inhibitor, GF-109203X, which prevents β-catenin ubiquitination, inhibits APC-mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner.** SW480 cells were transfected with empty vector or WT APC, LEF reporters, and pCMV-*Renilla* luciferase. 12 h posttransfection, cells were treated with various concentrations of GF-109203X. 12 h later, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

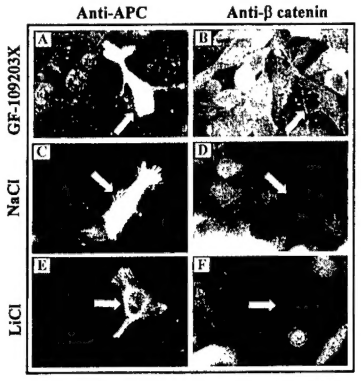

FIG. 6. **The bisindoylmaleimide-type PKC inhibitor, GF-109203X, but not lithium, reverses the APC-mediated down-regulation of β-catenin protein.** SW480 cells were transfected with WT APC and were treated with 5 μM GF-109203X (*A* and *B*) for 12 h as described in Fig. 5. 20 mM NaCl (*C* and *D*) or LiCl (*E* and IF) were added immediately following transfections and were present throughout the 24-h assay period to assure GSK-3β repression. Double immunofluorescent staining for APC (*A*, *C*, and *E*) and β-catenin (*B*, *D*, and *F*) was performed according to Munemitsu *et al.* (11, 40).

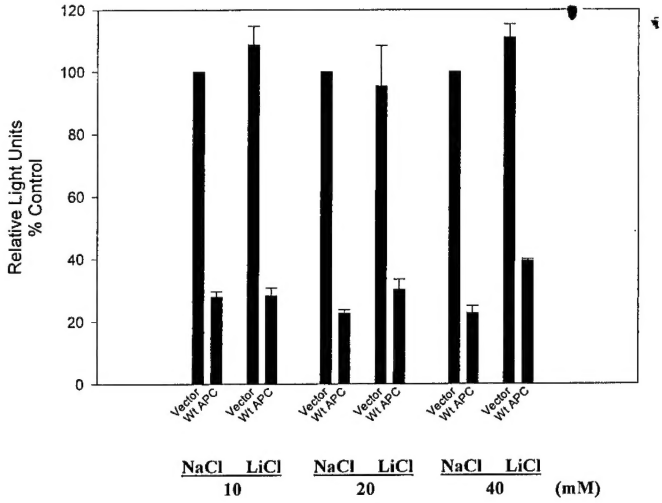

FIG. 7. **Lithium, an inhibitor of GSK-3β, does not significantly alter the ability of exogenous WT APC to down-regulate LEF reporter activity.** SW480 cells were transfected with empty vector or WT APC, LEF reporters, and pCMV-*Renilla* luciferase. Various concentrations of NaCl or LiCl were added immediately after transfection to assure GSK-3β repression. 24 h later, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

APC to down-regulate β-catenin-LEF signaling. The colon cancer cell line SW480 was transfected with empty vector or WT APC and treated with 10, 20, or 40 mM LiCl or NaCl for 24 h. The treatments were initiated immediately following the 5-h transfection period, and the cells were exposed to LiCl or NaCl throughout the 24-h assay period to assure GSK-3β repression. Fig. 6 shows that lithium does not alter the ability of WT APC to down-regulate β-catenin protein. Fig. 7 shows that lithium does not reverse the ability of WT APC to down-regulate LEF reporter activity in SW480 cells. Even at 40 mM lithium, a level well above that required to completely inhibit GSK-3β, exogenous WT APC continues to significantly down-regulate LEF reporter activity. These experiments were repeated in several different formats incorporating variations in the amount of WT APC transfected, duration of treatment with lithium, and timing of treatment initiation following transfections. Regardless of these variations, lithium does not inhibit the ability of exogenous APC to down-regulate β-catenin-LEF signaling in the colon cancer cells tested. Lithium treatment also leads to activation of AP-1-luciferase reporter activity in *Xenopus* embryos, consistent with previous observations that GSK-3β inhibits c-*jun* activity (46, 51). Concurrent AP-1 transactivation assays also confirmed that GSK-3β was inhibited in SW480 cells following treatment with lithium (data not shown). These results indicate that GSK-3β activity (the molecular target of lithium action, in the Wnt signaling cascade) is not required for the ability of exogenously expressed APC to down-regulate β-catenin. Recent data indicated that the role of GSK-3β may be to potentiate assembly of the APC·Axin·β-catenin complex (48). In our experiments, the high level of APC expressed in the transiently transfected cells may well drive complex assembly in the absence of GSK-3β activity. Indeed, in SKBR3 cells, lithium treatment causes the accumulation of cytoplasmic β-catenin and increases β-catenin-LEF signaling[2] (25).

Our observations suggest that one function of APC is to down-regulate β-catenin-LEF signaling via the ubiquitin-proteasome pathway. *In vitro* reconstitution experiments designed to explore β-catenin ubiquitination suggested the requirement of key components other than GSK-3β and APC.[2] During the course of this study there has been an explosion of data describing novel proteins, including Axin, Conductin, and Slimb·β-TrCP as regulators of β-catenin stability (47, 52–57). In *Drosophila,* loss of function of Slimb results in accumulation of high levels of Armadillo and the ectopic expression of Wg-responsive genes (56). Recently, the receptor component of the IκB·ubiquitin ligase complex has been identified as a member of the Slimb·β-TrCP family (39). Considering the increasing number of similarities between the regulation of IκB and β-catenin (25), it is tempting to speculate that like IκB, β-catenin ubiquitination occurs in a multiprotein complex that includes kinases, ubiquitin-conjugating enzymes, and co-factors. Context-dependent potentiation of this complex by GSK-3β and other serine kinase(s) may be regulated by DAG-dependent and -independent PKC activity, respectively. The challenge for future studies will be to determine the exact role of APC in this process.

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[2] V. Easwaran and S. Byers, unpublished observations.

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